# ANNUAL REPORT
# OF
# PROGRAM ACTIVITIES

## NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

## FISCAL YEAR 1973

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE NATIONAL INSTITUTES OF HEALTH

ANNUAL REPORT

OF

PROGRAM ACTIVITIES

U.S. NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Fiscal Year 1973

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service National Institutes of Health

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TABLES OF CONTENTS

ANNUAL REPORT

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

DIRECTOR
GEOGRAPHIC MEDICINE BRANCH

EXTRAMURAL PROGRAMS

COLLABORATIVE PROGRAMS
INFECTIOUS DISEASE BRANCH
TRANSPLANTATION AND IMMUNOLOGY BRANCH
RESEARCH RESOURCES BRANCH

LABORATORY AND CLINICAL RESEARCH PROGRAMS

SCIENTIFIC DIRECTOR
SUMMARIES AND PROJECT REPORTS

LABORATORY OF CLINICAL INVESTIGATION
LABORATORY OF INFECTIOUS DISEASES
LABORATORY OF VIRAL DISEASES
LABORATORY OF MICROBIOLOGY
LABORATORY OF BIOLOGY OF VIRUSES
LABORATORY OF MICROBIAL IMMUNITY
LABORATORY OF PARASITIC DISEASES
LABORATORY OF IMMUNOLOGY
ROCKY MOUNTAIN LABORATORY



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ANNUAL REPORT OF PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
JULY 1, 1972 THROUGH JUNE 30, 1973

The National Institute of Allergy and Infectious Diseases conducts and supports a broad program of research directed at helping medical science find improved ways of diagnosing, treating, and preventing allergic conditions and infectious diseases.

A major change this year was the curtailment of research training grants, fellowships and research career development awards in anticipation of the termination of Institute training programs. Research training grants and fellowships with commitments for future years are being continued at a diminishing level with no new appointments.

Only one organizational change occurred during the year. On May 1, the Geographic Medicine Branch which was formerly under the Office of the Director, NIAID, was incorporated into the Collaborative Research Program. Dr. Howard A. Minners, former Chief of the Branch, was appointed Associate Director for Collaborative Research and Dr. Robert J. Byrne as Assistant Associate Director. Dr. Earl S. Beck became Chief, Geographic Medicine Branch. Dr. John R. Seal remained the Scientific Director with responsibility for the Intramural Program and for coordination and planning of the Intramural and Collaborative Program with special reference to influenza and hepatitis.

The Institute activity for the support of scientific communications, and for the development of new and improved methods has continued at a level comparable to the previous year. The greater part of the support relates to the classical mechanisms of scientific manuscripts and scientific meetings. Encouraging progress has been realized in two feasibility projects for the development of newer communication techniques.

The creativity of research investigations is realized primarily through the publication of results in the archival scientific literature. During FY73 an estimated 2700 scientific manuscripts and research notes were published by NIAID grantees and another 196 from research contractors. Publications produced by the Institute's staff scientists totaled approximately 450. In addition, 116 technical or progress reports from research contractors were received and copies sent to the Department of Commerce "National Technical Information Service" from where they are available to the scientific community and the public.

The support of scientific meetings has continued at the same level in number of meetings sponsored but the level of support per meeting generally has been reduced. The reduction has been effected through provision of a subsidy rather than total costs for the attendee's travel and subsistence expenses. A trend has continued in the greater use of small specialized scientific workshops to concentrate attention on a defined aspect of a study,



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to gather and analyze new research data, and to determine progress and the next logical approaches in that research study.

The NIAID studies of new methods of communications between research investigators have continued with encouraging progress. The "Scientific Memoranda" afford fast, frequent and informal communications related to on-going research in defined, narrow fields. The progress reports, research notes, negative data and comments are exchanged while the investigations are being conducted and provide useful information months before the research results are available in the formal scientific literature. Six projects are now operational: Interferon Scientific Memoranda, Leprosy Scientific Memoranda, Hepatitis Scientific Memoranda, Hypersensitivity Disease Memoranda, Neisseria Treponema Scientific Memoranda, and Cell-free Immune Transfer Scientific Memoranda. As of May 1973, there were 801 scientists participating in these projects; 447 located in the United States and 354 in 49 other countries. Produced as annexes to the pertinent project were the annual Interferon Bibliography, and the Hepatitis Bibliography (1969-1972) which were produced from MEDLARS in collaboration with the National Library of Medicine.

In the satellite communications project studies are being conducted of the usefulness of this system for scientific communications. Initial efforts are devoted to adapting existing equipment and developing techniques for immediate voice and data transmission exchanges with and between isolated laboratories and field study units. Using relatively simple equipment (e.g. FM radio transceivers similar to those used in taxicabs) communications are possible over a broad geographic area wherein telephone communications are not possible or practical. For example, research scientists conducting field studies on a small South Pacific atoll or deep in a tropical jungle, can communicate directly with NIH scientists in Bethesda, Maryland. Through a telephone connection at the Bethesda or any other receiving point, the isolated investigators can talk with any specialists with whom they need to consult to aid their field investigations. By connecting to the system other available equipment, data sheets can also be transmitted.

In line with a new communications policy of the DHEW's Office of Public Affairs, the Institute's Research Information Section selected five research areas for special attention this year. Most of the projects initiated by the Section were in these five areas; for example:

Influenza - produced a color film featurette on NIAID research with Chincoteague ponies and released to local TV stations, network feeds, and USIA for overseas telecasts; held briefing for science writers on genetics of influenza virus; arranged for major articles in Science and JAMA on influenza research.

Hepatitis - updated viral hepatitis fact sheet and produced leaflet on hepatitis.

Venereal Disease -- produced fact sheet on venereal disease; prepared special report to the Congress on VD research (for the first time), and Information Office participated in 3rd International VD Symposium.

Asthma and Other Allergies -- produced an exhibit on Allergic Disease research, highlighting the Institute's 10 Allergic Disease Centers; assisted in the American Academy of Allergy convention press room; prepared pamphlet on "Asthma"; produced leaflet on "Food Allergy"; arranged interview for 5-page article on allergy in U.S. News and World Report.

Clinical Immunolgy -- prepared brochure on "Clinical Immunology, An Introduction"; reprinted and distributed two articles on immunology written by Harold Schmeck after they appeared in the New York Times.

The success of "Allergy Research, An Introduction", released early in the fiscal year, made it necessary to go "back to press" when Institute and Superintendent of Documents inventories were depleted. The Section distributed more than twice as many publications this fiscal year as last. In addition to the new and updated publications mentioned above, a new fact sheet on Rocky Mountain Spotted Fever was produced and fact sheets on Pneumococcal Infections and Malaria were updated. The Section now distributes 18 fact sheets.

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GEOGRAPHIC MEDICINE BRANCH

SUMMARY

The Geographic Medicine Branch was created in the NIAID in 1968 to manage three international programs: The United States - Japan Cooperative Medical Science Program, the International Centers for Medical Research (ICMR), and the International Research Career Development Program (IRCDP). The IRCDP has since been modified and incorporated within the ICMR Program.

I. The United States - Japan Cooperative Medical Science Program

Background

The U.S.-Japan Cooperative Medical Science Program was the product of a January 1965 meeting of the two heads of state of the United States and Japan. The mutual concern for the health and well-being of people in underdeveloped countries prompted the two leaders to agree to undertake jointly a cooperative medical research effort focused upon health problems of recognized importance in Asia. The relevant regions in Asia are not specifically defined, but are generally understood to include the Republic of Korea on the north, India and Pakistan to the west, and other adjacent nations in the broad Pacific basin.

The implementation of this Program involved the time and effort of prominent medical scientists of both countries to identify and define specific parameters of the disease categories to be included in the new Program. From this group of scientists evolved the Joint U.S.-Japan Cooperative Medical Science Program Committee which meets annually, alternating between Japan and the United States, to review the Program's objectives, operations and accomplishments, and to initiate new management policies and procedures. The next meeting of the Committee, the ninth, will be held at the National Institutes of Health on July 26 and 27, 1973.

The United States' Delegation to this Committee is appointed by our Secretary of State, and Japan's Delegation by the Japanese Ministry of Foreign Affairs. The Delegations advise their respective governments on the scope, direction and other broad aspects of the Program, and develop review procedures to assure fulfillment of the purposes for which the Program was established.

In Japan, the Program activities are the responsibility of the Ministry of Health and Welfare and the Ministry of Education. In the United States, the operational responsibility for the Program has been delegated to the National Institutes of Health, Department of Health, Education and Welfare. The United States and Japan maintain separate secretariats.

The Program is concerned with the following diseases or categories of diseases: 1. cholera, 2. leprosy, 3. malnutrition, 4. the parasitic diseases, schistosomiasis and filariasis, 5. tuberculosis, and

6. selected viral diseases (rabies and dengue-hemorrhagic fever). A seventh Panel focused on selected health effects of environmental pollutants was initiated during 1972.

For each of the seven areas selected for study, ten-member joint Panels, consisting of five scientists from each nation, were established to delineate more specifically areas for mutual cooperation and to carry on Program activities. Briefly summarized, these activities may be grouped into the following three categories: support of research, joint scientific conferences, and exchange of persons. In addition, each Panel prepares an annual report of progress for review by the Committee. The scientific meetings of the joint Panels alternate annually between locations in the United States and Japan; in 1973, all joint meetings will be held in the United States.

The overall U.S.-Japan Cooperative Medical Science Program operates within a bilateral government framework. Nevertheless, it may involve scientists and facilities in third countries, and/or collaboration with international or other organizations, with mutual approval of both Delegations.

The United States and Japan each support the cost of their own scientific projects and meetings. Research by U.S. scientists is funded by the United States and that of Japanese scientists by Japan. Exchange of scientists between the two countries is encouraged as a matter of principle and is supported by official or unofficial agencies of either country. The U.S. does not fund research in Japan nor grant fellowships to Japanese scientists under this Program, nor does Japan fund U.S. research or fellowships for Americans as a component of their Program-sponsored activities.

The National Institutes of Health provides:

1. support of scientific projects

2. organization, funding, and conduct of scientific meetings held in the United States

3. financial support to the United States Panel members and consultants attending meetings related to the Program

4. staff and operating costs of the U.S. Secretariat

This support is based upon a specific delegation of Presidential authority under Public Law 86-610, the International Health Research Act of 1960, and on the availability of funds.

The Japanese Ministries of Health and Welfare and of Education assume a similar responsibility for their counterpart activities, i.e. research projects, meetings held in Japan, Japanese Panel members and consultants, and for the Japanese secretariat.

## Highlights of the Past Year

### CHOLERA

The cholera program sponsored a round table discussion on the epidemiology of cholera and international control in view of the recent spread of the disease from Southeast Asia to Africa and Europe.

Special emphasis has been placed upon clarifying the roles of antitoxic and antibacterial (somatic antigen) immunity in cholera. Progress has been made in achieving purification of antigens found in the vibrio cell membrane. A major activity is the continued support of the development and testing of a cholera toxoid. Since the formalinized toxoid was found to be unstable, a second attempt at producing toxoid by using glutaraldehyde appears to be satisfactory. The material is antigenic with aluminum adjuvant, completely stable and its safety has been proven in animals and volunteers, and the duration of the resulting immunity is being monitored.

Studies of the mechanisms of action of purified cholera toxin at the molecular level have provided more evidence that the manifestations of toxic activity in the gut as well as in a number of other tissues are associated with elevated adenyl cyclase activity. A specific ganglioside has been shown to act as a potent toxin deactivator and may function as a cell membrane binding site. The relationship between these two observations should be an important focus for future investigation. Studies have been carried out on mutants of Vibrio cholerae which are devoid of cyclic AMP activity in an attempt to clarify the role of the cyclic AMP system in vibrio cells. The use of these mutants should help to elucidate the mechanism of toxin action.

Considerable new information has emerged on the distribution and pathogenesis of diarrheal disease caused by enterotoxin-producing Escherichia coli. The methods of assay and the properties of E. coli enterotoxins are also receiving increasing attention. It now appears that cholera antitoxin will inactivate one E. coli toxin associated with diarrhea. The cholera program is actively supporting this work, together with studies on other non-cholera enterotoxins, with the view that an understanding and control of the enterotoxin-mediated diarrheal diseases will best be served by a unified approach to their natural history.

### LEPROSY

This past year, the U.S. Leprosy Panel has seen advances in pharmacology, immunology, and cultivation. In pharmacology, the early effects of the three-year mass chemoprophylactic treatment for leprosy with acedapsone (DADDS) in the isolated Pingelapese population are now being seen. DADDS is a repository type derivative of the standard antileprotic drug, dapsone, (DDS). The findings of the second annual surveillance of the population are as follows: three persons developed leprosy two years after the DADDS injections were discontinued in the general population. The expected number



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of annual cases in the untreated population is twelve. Persons coming down with leprosy had received less than ten injections. There have been no cases of leprosy observed in people who received all fifteen injections. Two or three injections of DADDS does not appear to prevent the onset of leprosy in persons about to develop the disease, however, three or four injections of DADDS suppresses the onset of the disease for the duration of the injections and for about 6-12 months thereafter. From this it would appear that there is a threshold number of injections of DADDS for leprosy prophylaxis.

New cases of leprosy are being treated with DADDS and surveillance of the entire population is planned through 1980. If the DADDS chemoprophylaxis proves to be effective over the long term, DADDS will significantly facilitate the therapy of leprosy patients in endemic areas with limited medical and public health capacity.

In continuing studies of the metabolism of DDS and the phenotypic classification of subjects according to acetylation characteristics, recent results suggest some association between M. leprae resistance and intermediate and fast acetylators. An increased rate of clearance of DDS and MADDS from the plasma was also related to DDS resistance.

The chromatographic-fluormetric procedures for the measurement of DDS, MADDS, and DADDS have been improved. It is now possible to detect as little as 0.1 to 0.2 ng/ml of plasma. This can be accomplished in one run, instead of the two previously required, with a 50% reduction in total analysis time.

Two chemotherapy trials (B663 II and rifampin I) have been completed, and the final results are presently being written up. The data of the B663 II trial of 48 patients demonstrated that the daily regimens were more effective than the intermittent ones. The skin pigmentation side effects and ENL (erythema nodosum leprosum) reactions will be described fully in the publication.

In the rifampin I trial, the efficacy of rifampin against M. leprae was compared with DDS in untreated lepromatous patients. The viability of M. leprae from biopsy specimens was studied by the mouse footpad test.

The results show that biopsy specimens from patients treated with daily rifampin were negative in three months, whereas the specimens from the DDS treated group were not negative until six months. The therapeutic effect of intermittent rifampin and DADDS is now being studied. This type of rifampin regimen is more desirable, since rifampin is expensive and effective intermittent therapy is more practical in underdeveloped countries where leprosy is endemic. The combined therapy might also reduce the likelihood of patients developing drug resistance.

Progress in the area of immunology has continued. Encouraged by the results of single injections of transfer factor into lepromatous patients, the U.S. Leprosy Panel has designed a transfer factor protocol for further studies of the immunologic deficiencies noted in leprosy.

Encouraging results with the thymectomized-irradiated mouse as an animal model for leprosy research continued throughout the year; however, the problem of reduced longevity has not been solved in this immunological defective animal.

An efficient method for the production of antilymphocyte serum (ALS) has been developed, and a means for testing the potency has been standardized. This product is available to approved investigators through a service contract.

Some encouraging reports have come from the continued efforts to cultivate the leprosy bacilli. There is evidence that M. leprae grows readily in some armadillos, producing a disease condition similar to that in man. It is very possible that this animal could be a valuable source of biopsy material for leprosy research. Human skin biopsy specimens with large numbers of M. leprae are now provided by the U.S.-Japan Program.

MALNUTRITION

Additional data confirm earlier reports that severe malnutrition during the first two or three years of life, even though dietary adequacy is later provided, has a lasting detrimental effect upon intelligence and behavior in later life. It is anticipated that highly sophisticated neuropsychiatric and neurophysiologic methodology currently being developed and applied will provide the basis for more exacting quantification of brain functions and allow more precise evaluation of the influence of early malnutrition thereon.

There has been a dramatic fall in morbidity rates from infectious diseases observed in children receiving both nutritional supplements and medical care. This was not seen when medical care alone was provided. Better control of infections which so commonly thwart recovery from severe malnutrition in infancy are under careful study.

Also under investigation in several countries is the prevalence and frequency of iron and protein deficiency in children and of folic acid deficiency in pregnant women. Other research in progress endeavors to explain, and find means of correcting, the poor absorption of iron from cereal grains.

Work continues in an attempt to incorporate a high level of protein into commercially produced rice in Southeast Asia. The biological quality of rice protein is fairly high and an increase in the protein level in rice would go far toward alleviating protein undernutrition in countries for whom rice is the mainstay of subsistence. Based on recent breeding work involving progeny of successful crosses between six high-protein strains and $IR_8$, the consensus is that the protein content of the new rice is a genetically determined characteristic. Four of the six strains contain 10 1/2 per cent protein which can be pushed to 12 per cent under optimal conditions of growth. Breeding experiments are in progress to incorporate into the strains resistance to certain plant diseases. The human nitrogen

balance studies using one of the high protein rice varieties in adults were so encouraging that the same type of study will be done in rapidly growing infants and preschool children in the Philippines.

## PARASITIC DISEASES

Research under the Parasitic Diseases Panel is confined to two parasitic infections of very great importance in Asian Countries - schistosomiasis and filariasis.

### Schistosomiasis

*In vitro* cultivation of schistosomula to the preadult stage of *S. japonicum* has been successful and should aid in the development of anti-schistosomal drugs.

Studies have continued in gaining an understanding of the mechanism of immune response in schistosomiasis. It has been shown that two anti-schistosomal drugs, tartar emetic and niridazole, suppress the schistosome egg granuloma *via* anti-inflammatory action. The role of anti-DNA antibodies in glonerulonephritis associated with human and experimental schistosomiasis is being investigated.

With the development of aseptic techniques a design of suitable media, work toward the objective of establishing a cell line of the snail host of *S. mansoni* has progressed. In addition, sporocysts of *S. mansoni* have undergone development to cercarial embryos in these *in vitro* snail cultures.

Chemotherapeutic studies have included development and testing of synthetic antischistosomal agents. Structural and conformational similarities of two effective compounds have led to a proposal that antischistosomal activity has the following requirements: a 5-nitroheterocyclic ring linked in the 2-position to a nitrogen of low basicity *via* a vinyl group. Another study has shown that tubercidin administered after its adsorption on to host red blood cells was schistosomicidal against both *S. japonicum* and *S. mansoni* in monkeys.

### Filariasis

Further work has shown that the gerbil is the most useful rodent host for studies with the lymphatic-dwelling filarial worms. Base-line studies are continuing with the aim of using this host for evaluation of new filaricidal compounds.

Biochemical studies of several filarial species indicate that a few metabolic pathways are sufficiently different from the analogous mammalian counterparts to be potentially exploitable by appropriate kinds of drugs.

Efforts have continued to develop a vaccine composed of infective filarial larvae which have been suitably attenuated *via* irradiation.

Histopathological studies of Brugia pahangi in dogs have shown that both humoral and cellular response mechanisms rise and fall in intensity in correlation with molting and initiation of microfilarial deposition.

Studies of chemotherapeutic agents are being carried out to determine which metabolic systems are affected by specific compounds. Further critical research on filarial physiology needs to be done.

A workshop in "Factors affecting the competence of diptera as vectors of arboviruses and filariae" included discussions of the genetics of mosquito susceptibility, specificity and development of filariae in arthropod hosts, and population dynamics of mosquitoes.

## TUBERCULOSIS

In the past year significant advances have been made in the field of tuberculosis research. Much of this has been due to the cooperative efforts of a number of investigators and the availability of standardized, highly characterized mycobacterial materials produced through service contracts of the U.S.-Japan Program. Mycobacterial cell extracts and culture filtrates have been produced and disrupted under specified conditions. These materials will be used to isolate specific antigenic fractions which will eventually be studied for in vivo and in vitro activity and used in immunological research. Mycobacterial products such as BCG, PPD, and cell wall fractions are recognized as promising immunological adjuncts in the treatment and prevention of mycobacterial diseases as well as carcinogenic and immune dificient diseases.

A reference system of mycobacterial antigen and antibody serum is now available. The system has been standardized and characterized by immuno-electrophoretic procedures.

Progress has been made in evaluating existing animal models for the study of tuberculosis vaccines. A sub-human primate model has been studied using aersolized BCG vaccination and followed by aersolized challenge. Dose-response data are now being collated.

## VIRAL DISEASES

Research under the Viral Diseases Panel is limited to studies on rabies and on arboviruses with emphasis on dengue and the pathogenesis of hemorrhagic fever.

### Rabies

Studies on antigenic relationships have shown that there are at least six different virus types from Africa which are serologically related to rabies virus. The newly studied rabies - relatives add to the serotypes available to test the capacity of these strains to breaden the immunity to rabies virus.

Survival time of infected mice was significantly prolonged by several methods of immunosuppression indicating that an immune mechanism is involved in the pathogenesis of at least one of the rabies serogroup viruses.

Work has continued on the isolation and characterization of the protective antigens of rabies virus. In addition, the role of adjuvants as augmenters of immunological response is being studied with the goal of administration of a massive dose of purified antigen attached to mineral adjuvant.

Also being developed are improved methods for the inactivation of rabies virus without significant loss of immunogenicity. Included were studies of the kinetics of beta-propriolactone degradation, refinement of assays for residual live virus and actual inactivation of virus by both formalin and beta-propriolactone.

Dengue

Various procedures have been evaluated for potential use in the diagnosis of dengue and in understanding its epidemiology. Area-treated CF antigen and standard sucrose-acetone antigens and dengue virus were compared. The former was more type specific but less sensitive than the latter.

The first comparative electron microscopic study of the four dengue virus types, propagated in vitro, has been completed. All dengue viruses appear to be spherical as they mature and the particle size of the four types is the same. The sequential stages of viral development and release for the four types of dengue virus, in infected cells, are quite similar.

Continuing pathological studies have involved the search for dengue antigen and immune complex in biopsied tissue of kidneys and skin and in autopsy material obtained from patients with dengue hemorrhagic fever. The blood vessels of the skin were the only sites where dengue antigen could be found in significant amounts in conjunction with complement. Circulating leucocytes were also found to harbor dengue antigen. Evidence has thus been obtained to support the hypothesis that the pathology and pathogenesis of vascular changes are related to immunological reaction.

Attempts are being made to establish an in vitro model of dengue infection. In vitro formed virus-antibody complexes are being studied to determine the effect of dengue virus of Raji cells in the presence and absence of antibody and complement. The hypothesis is that complement activation may be triggered by a simultaneous production of large amounts of virus and anti-dengue antibody during secondary dengue infection.

Environmental mutagenesis and carcinogenesis

There is expanding evidence that human health will be increasingly influenced by chemical pollutants introduced by man into his environment. The purpose of the Panel is to develop better methods to detect potential mutagens and carcinogens and to evaluate existing methodology.

Research Conferences and Workshops:

In addition to program elements involving research grants and contracts, the U.S.-Japan Cooperative Medical Science Program includes a provision for regular scientific conferences and workshops. These include joint meetings with Japanese counterpart scientists as well as special conferences for American investigators working in the scientific areas of special interest to a given Panel. These workshops and conferences serve to stimulate interest and enthusiasm which leads to the development of new scientific approaches to the seven disease categories within the Program, and they also facilitate the timely exchange of research data, methods and protocols. The Joint U.S. and Japanese Panels meet annually, alternating between locations in the United States and Japan. A notation of conferences and workshops sponsored during FY 1973 follows:

| Meeting Title and Location | Sponsoring Panel |
|---|---|
| July 18-20, 1972<br>Joint Parasitic Diseases Conference<br>Hiroshima, Japan | Parasitic Diseases |
| August 20-21, 1972<br>Cholera Round Table<br>Kumamoto, Japan | Cholera |
| August 22-23, 1972<br>Joint Cholera Conference<br>Tokyo, Japan | Cholera |
| August 21-23, 1972<br>Joint Tuberculosis Conference<br>Tokyo, Japan | Tuberculosis |
| August 24-25, 1972<br>Joint Committee Meeting<br>Symposium<br>Tokyo, Japan | |
| August 25-27, 1972<br>Joint Environmental Conference<br>Tokyo, Japan | Environmental |
| September 13, 1972<br>Filariasis Workshop<br>New Orleans, Louisiana | Parasitic Diseases |
| September 18-20, 1972<br>Joint Viral Diseases Conference<br>Sapporo, Japan | Viral |

| Meeting Title and Location | Sponsoring Panel |
|---|---|
| September 21, 1972<br>Joint Cholera and Malnutrition Meeting<br>Bethesda, Maryland | Malnutrition/Cholera |
| September 28, 1972<br>Leprosy Workshop<br>Atlanta, Georgia | Leprosy |
| November 8-9, 1972<br>Joint Leprosy Conference<br>Tokyo, Japan | Leprosy |
| November 28-29, 1972<br>Joint Malnutrition Conference<br>Kyoto, Japan | Malnutrition |
| January 4-5, 1973<br>Joint Meeting of the Parasitic and Viral Panels<br>Berkeley, California | Parasitic/Viral |
| January 25-26, 1973<br>Joint Sub-Committee Meeting<br>Hilo, Hawaii | |
| June 25, 1973<br>U.S. Delegation Meeting<br>State Department<br>Washington, D.C. | |

II. The International Centers for Medical Research (ICMR) Program

Program Description:

In response to the expressed desire of the Congress (P.L. 86-610), a program for the International Centers for Medical Research was established in 1960 by the National Institutes of Health "to advance the status of the health sciences in the United States and thereby the health of the American people" by expanding collaborative research between U.S. universities and selected foreign institutions and investigators.

The four grants funded under this Program are awarded for five years with continued support contingent upon program review at the end of each year of operation. One of the most important features of this program is that of long-term support, as contrasted with the transiency which has characterized a number of other international projects. This is a necessary ingredient for the full development of university careers in science which will effectively utilize the international medical experience of individual scientists. A major advantage of this program is that it provides opportunities for established and highly competent investigators to exploit

valuable research opportunities abroad without losing their university affiliations and faculty appointments. The long-term support of the ICMR's does not represent a form of foreign aid. Rather the primary objective is a collaborative effort in biomedical research of specific relevance to the health of the American people and to the U.S. scientific community. The four current ICMR grants were renewed for an additional five years beginning in FY 1970, and at the same time support was phased out to the LSU Center in San Jose, Costa Rica.

Purpose and Scope:

The ICMR program has as its principal objective the provision of high quality research and scientific opportunities for Americans in the broad fields of geographic medicine, and in response to the special opportunities existing within each ICMR framework. The length of the research period overseas is a discretionary concern of the ICMR program director, and is obviously determined by both the interests of the prospective investigator and the specific opportunities available within the given ICMR unit. Consistently, the emphasis is upon the quality rather than the quantity or duration of the research programs.

The aggregate ICMR units are serving increasingly as a national resource for utilization by senior as well as less experienced scientists to create a comparatively modest pool of investigators with a sustained career interest in international or geographic biomedical research. In this regard, the ICMR program directors are encouraged to establish a selective, interdisciplinary scientific program. Their activities encourage and accommodate a number of parent university departments other than the department which primarily sponsors the ICMR operation. Particular advantage is taken of special strengths within the overall parent university, including opportunities in health-related fields outside of the classical confines of tropical medicine.

During FY 1971, the NIH initiated a new program element within the overall ICMR framework. Designated as the ICMR Research Associate Program (IRAP), this centrally coordinated activity is specially designed to recruit investigators on a national basis and specifically from universities that do not have an ICMR.

As far as is possible, the ICMR core grant is used for research support at the off-shore ICMR site rather than at the parent, domestic university. In this regard it is accepted that some preparation preliminary to an overseas assignment is frequently necessary or desirable, and as many items of equipment and supplies as feasible are purchased in the United States.

Program:

The four institutions participating in the ICMR program at present, and their respective areas of research interests are as follows:

Johns Hopkins University (Baltimore, Maryland)

Program Director: Dr. Frederik B. Bang

Johns Hopkins University with the participation of the School of Hygiene and Public Health and the Department of Medicine is collaborating with the Calcutta School of Tropical Medicine, the All-India Institute of Hygiene and Public Health, and the Postgraduate Institute for Medical Education and Research in Calcutta, India. During the past year scientific activities have also been initiated in Kathmandu, Nepal, and Dacca, Bangladesh in anticipation of an eventual move from Calcutta. Program emphasis is directed toward the epidemiology and management of human diseases of large populations and the ecology and behavior of animals which live in comparatively close association with man.

Studies are in progress on the year-round distribution of respiratory and enteroviruses in normal children in rural communities. Other research includes the development of antigens for skin tests in leprosy, and the ecology of the economically important bandicoot rat (B. bengalensis).

Intensive biochemical research in cholera is also in progress. This work includes antibiotic and oral therapy, cholera in children, comparison of classical and El Tor cholera, and a number of very significant experimental pathophysiological studies.

University of California (San Francisco, California)

Program Director: Dr. J. Ralph Audy

The University of California with the participation of the Hooper Foundation, the School of Public Health, and the Medical School, is collaborating with the Institute for Medical Research, Kuala Lumpur, Malaysia, and the University of Malaya. This International Center continues to place strong emphasis on infectious diseases transmitted from animals to man. Because of the long standing research interest and experience in studying the fundamental problems of human ecology at the University of California, a collaborative program has been developed embracing medical zoology, microbiology and parasitology. The scope of this collaborative endeavor has now been expanded to include related socio-cultural research and medical genetics.

Long-term research projects in Malaysia and California are based on three general laboratory programs: the Arbovirus Research Unit, the Parasitology Laboratory, and the Medical Anthropology and Rural Health Research project.

The arbovirus research is focused on the epidemiology and ecology of dengue virus and the relationship of this virus to mosquito-borne hemorrhagic fever. A jungle reservoir (monkey) has recently been domonstrated for dengue. The parasitological research is based on studies of trematode ecology and interactions of various parasites in the snail host and the ecology of Malayan pentastomes or tongue worms. The snail trematode antagonism studies are directed toward the biological control of schistosomiasis, and this ICMR

represents a leading world resource for this important field research. Investigators from this ICMR have also studied the histopathology of host reaction to acarine parasites, the relationship of monkeys to human malarial infection, and jungle cycles of scrub thphus, Q fever, and tick typhus.

Abundance, distribution, life cycles and ectoparasites of rodents closely associated with man have also been studies. Work on the possible role of the Indian fruit bat as a reservoir of human viral infections has been completed. Other field studies include the investigation of bacteria and viruses pathogenic for man in monkey populations. Socio-cultural studies concerned with Malayan attitudes toward health and nutrition and the influence of native and western practitioners of medicine upon these attitudes have been undertaken by a number of variously trained, medically oriented social scientists.

University of Maryland (Baltimore, Maryland)

Program Director: Dr. David F. Clyde

The University of Maryland with the participation of its School of Medicine, and collaborating with the Institute of Hygiene, Lahore has undertaken a varied research program in West Pakistan. Comprehensive studies of arthropod-borne diseases have been undertaken. During FY71, a field unit in Salvador (Bahia), Brazil was established at the Federal University of Bahia to study insect vector pathology as a tool for biologic control of malaria. Additional studies are planned in the area of schistosomiasis and vector genetics. Because long-term stability has become increasingly difficult to insure in Pakistan, it is likely that the University of Maryland will eventually relocate their ICMR from Pakistan to Brazil.

More recent research in Pakistan includes studies of mosquito-borne viruses in man. In addition, a detailed investigation of several different clinical entities, e.g. bleeding disorders in humans, has been recently completed. Research on immunity in tuberculosis and on the relationship between cirrhosis, hepatitis-associated antigen, and viral hepatitis has also been completed.

Mosquito genetic investigations of a comparatively unstudied vector of Japanese encephalitis virus have been of unusually high scientific merit and promise. Knowledge about the genetics of this mosquito, Culex tritaeniorhnychus, now approaches the abundance and high quality of information previously known about two other mosquito vectors of human disease. This new information has been sought to establish a scientific basis for the ultimate biologic control of human Japanese encephalitis. A new "compound chromosome" technique has been developed and is under consideration for early field application on a limited scale.

Tulane University (New Orleans, Louisiana)

Program Director: Dr. Paul C. Beaver

Tulane University, with the participation of the Department of Tropical Medicine and Public Health, other departments of the Medical School, and the Department of Anthropology and Sociology, is collaborating with the Universidad del Valle, Cali, Colombia. The research work is in two general areas: causes, prevalence and distribution of human diseases with human relevance, and physical environments which determine ill or well-being.

Parasitic diseases research has continued to focus principally around the following diseases: trypanosomiasis, malaria, filariasis, and amebiasis. The local epidemiology of Venezuelan Equine Encephalitis has been studied and documented following the recent epidemic. The Virus Group also has a continuing interest in rabies. A comprehensive clinical evaluation of bacterial meningitis treatment regimens is in process. Finally, the multi-disciplinary study of frustrated suicides has recently been continued and refined. New projects are concerned with maternal and child health, family planning, and include health services research.

GEOGRAPHIC MEDICINE BRANCH

CONTRACT NARRATIVES

U.S.-Japan Program

JEFFERSON MEDICAL COLLEGE (NIAID 73-2502) . . . . . Cholera
(formerly NIH 70-2188)

Title: Vibrio Reference Laboratory

Project Director: Dr. Harry L. Smith, Jr.

Project Officer: Dr. Carl E. Miller

Objectives: The objectives of this contract are: 1) to maintain a reference collection of V. cholerae and other vibrios and to supply these strains upon request, 2) to receive and identify serologically new vibrio isolates, and 3) to prepare and distribute to qualified investigators cholera typing antisera.

Major Findings: The contractor meticulously maintains a reference library of some 5000 cultures of V. cholerae and other vibrio-like strains. During the past year, 141 strains were received and 71 strains were supplied to investigators. Both of these figures are higher than last year. Also, 88 vials of diagnostic sera were provided as requested. This is a reduction from 632 vials last year. The total number of tests applied to each strain of V. cholerae was increased from 8 to 23. All new strains are characterized by these tests and 568 of the repository strains have also been characterized by these tests.

The diagnostic sera from sheep is still being evaluated under field conditions and no new report is available. The titer remains slightly lower than rabbit sera, but the volume obtained more than compensates for this reduced titer. The serum paper disc method of identification is also being evaluated in the field.

Significance to NIAID Program and Biomedical Research: It is important in cholera research to have a laboratory which possesses the competence and the expertise to identify accurately and to differentiate V. cholerae from other enteric organisms. The need for reference cultures and typing sera is important and essential to an active research program. Many investigators involved in the seventh cholera pandemic have used and depend upon the service and reagents of this laboratory.

Proposed Course of Project: The contract will remain in force as long as there is a demonstrated need for such a service.

Date Contract Initiated: September 1972

Current FY Funding Level: $35,450

THE JOHNS HOPKINS UNIVERSITY (NIH 70-2051) . . . . . Cholera

Title: Assays of Cholera Toxins and Toxoids in (A) Dogs and Rabbits and (B) Other Assay Systems

Project Director: Dr. Nathaniel F. Pierce

Project Officer: Dr. Carl E. Miller

Objectives: This contract is to provide a service laboratory for the broad-based cholera research program. The primary objectives include the maintenance of laboratory and experimental animal facilities for the evaluation of purified cholera toxins as immunizing agents against cholera, the development of improved assay techniques for the measurement of cholera toxoid and antitoxin activity, and the evaluation of immunizing techniques designed to achieve optimal antitoxin-mediated protection against cholera.

Major Findings: The glutaraldehyde toxoid has proven to be protective and safe in dogs. The results to date indicate 75-78% protection against lethal disease when dogs were challenged two weeks after 1) two parenteral doses of toxoid plus aluminum adjuvant, separated by 28 days or 2) one parenteral dose of toxoid plus adjuvant followed in 28 days by ten oral doses of toxoid. No adverse toxoid effects were noted. The results suggest that parenteral or combined parenteral oral immunization with this material may be protective against cholera in man. Tests on the duration of immunity in dogs are in progress.

Two systems for measuring cholera antitoxin have been carefully compared. They are, capillary permeability in rabbit skin and secretatory response of rabbit intestine. Antitoxin in serum and intestinal washings was measured in both systems. Similar titers were obtained except in specimens with low titers which were detectable only in the intestinal system. These studies provide a basis for comparison and indicate their usefulness in studying the role of local antibody in antitoxic protection against cholera.

The role of antitoxin in the intestine derived from three sources was studied. Intestinal antitoxin was demonstrable after 1) active parenteral immunization with toxoid, 2) passive intravenous immunization with homologous antiserum, and 3) after intraintestinal "boosting" with toxoid. In the latter situation, IgG and IgA were responsible for the antitoxic activity but only IgG was responsible in the passive immunization. These studies support the possibility that oral immunization or boosting may be effective immunizing procedures against cholera.

Significance to NIAID Program and Biomedical Research: This facility serves as a central testing laboratory for other investigators involved in cholera research. The service will provide uniform and rapid testing of submitted samples, thereby facilitating and expediting research toward the ultimate goal of developing an improved cholera toxoid.

Proposed Course of Project: It is anticipated that the contract will remain active until Program achieves or changes its guidelines.

Date Contract Initiated: December 1969

Current FY Funding Level: $79,220

THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK (NIH 70-2091)
. . . . . Cholera

Title: Establishment of a Laboratory for the Evaluation Measurement of Cholera Toxins, Antitoxins, and Toxoids

Project Director: Dr. John P. Craig

Project Officer: Dr. Carl E. Miller

Objectives: Establishment of a laboratory to perform titrations of cholera toxins permeability factor (PF), antitoxins, and antitoxin-combining-power on samples of toxins, toxoids or sera. Also, to assay the immunogenicity of cholera toxoids by the mouse protection test, and the residual toxicity by mouse inoculation.

Major Findings: The contractor has continued to test cholera toxins, toxoids and sera from the manufacturer and other investigators working with the new vaccine. Tests in mice have demonstrated that two doses of the glutaraldehyde toxoid produce an antitoxin level similar to two doses of the formalinized toxoid. And there is no evidence of toxin in the glutaraldehyde toxoid as was found in the formalin treated toxoid. The glutaraldehyde toxoid is now being tested in human volunteers.

The testing of 25 strains of V. cholerae, from Dr. Harry Smith's collection, for toxin production has revealed two strains which appear to be non-toxin producers. These strains will be very important for genetic studies related to the source of enterotoxin from cholera vibrios.

Significance to NIAID Program and Biomedical Research: The contractor will fulfill program need for comparative testing of materials under standard conditions for other research laboratories cooperating in the cholera program.

Proposed Course of Project: The contract will remain active until Program either achieves or changes its guidelines.

Date Contract Initiated: April 1970

Current FY Funding Level: $88,070

WYETH LABORATORIES INC. (NOI AI 32514) . . . . . Cholera

Title: Purified Choleragenic Toxins and Vaccine (toxoid) Production

Project Director: Dr. Benjamin Rubin
Dr. Alan Bernstein

Project Officer: Dr. Carl E. Miller

Objectives: The contractor will seek to convert purified toxin of standard quality to stable toxoid with optimal immunogenicity. Residual somatic antigen, if any, will be quantified by various tests. The techniques developed will be adapted to large scale production of toxoid suitable for a field trial. Practical adjuvanting methods will be developed in preparing test lots for clinical evaluation.

Major Findings: Further purification of cholera toxin by chromatographic procedures has produced a product with no detectable somatic antigen. Protamine-aluminum chloride adjuvant appears suitable for immunological enhancement of the toxoid. Even with this adjuvant, extremely low levels of vibriocidal antibody were obtained. The pilot lots of glutaraldehyde toxoid have passed the safety and potency tests in animals, and are now being tested in selected volunteers.

Processing of the toxin has been standardized and scaled to production levels. Approximately 30 grams of toxin will be produced, purified, converted to toxoid, tested and packaged for the field trial. Long term stability tests have been initiated.

Significance to NIAID Program and Biomedical Research: The Program represents a collaborative effort of the Cholera Advisory Committee and the U.S.-Japan Program to develop an improved cholera vaccine using detoxified toxin. Also testing of the toxoid in the field will help establish the present concept that known toxins in the field are responsible for lethal diarrhea, and that induced antitoxin can protect.

Proposed Course of Project: Continuation with incremental funding to produce the toxoid for field trial.

Date Contract Initiated: February 1973

Current FY Funding Level: $177,220

CENTER FOR DISEASE CONTROL (IAA-72-9909) . . . Leprosy

Title: Studies on the Pharmacology of Leprosy and the Use of Immunosuppressed Mice in Leprosy Research

Project Director: Dr. Charles C. Shepard

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor will continue to study the activity of DDS and other anti-leprotic drugs using a pharmacokinetic method and employing the mouse footpad technique. Tests for the activity of drugs at different times during the growth period, with known drug dosages, will be used. In this manner drug effectiveness (bactericidal effect) will be determined. The contractor will also continue studies on the use of immunologically suppressed mice. Thymectomized irradiated and ALS treated mice will be investigated.

Major Findings: The contractor has found other drugs besides DDS and B663 to be bactericidal in the mouse footpad test system: Sulfadimethoxine, ethionamide, rifampin, streptovaricin and cephaloridine. Preliminary results indicate that rifampin is also effective in treating leprosy in man. However, the high cost of this drug may preclude its extensive use. Most antimalarial drugs studied were not active as antileprotic agents. Combination drug tests are now in progress utilizing the mouse footpad system, and these data will be particularly useful in studying the apparent emergence of bacterial resistance to DDS.

Significance to NIAID Program and Biomedical Research: By using kinetic methods of analysis, correlated with the mouse footpad technique, the effectiveness of drugs can be investigated in laboratory models before initiating clinical studies.

Proposed Course of Project: This project will be continued to investigate other drugs which appear to be effective against the leprosy bacillus.

Date Contract Initiated: May 15, 1967

Current FY Funding Level: $75,000

HARVARD COLLEGE (NIH 70-2285). . . Leprosy

Title: Studies of Immunosuppression in Adult-thymectomized Mice Treated with Antilymphocyte Serum

Project Director: Dr. Anthony Monaco

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor proposes to study and define the course, duration and degree of immunosuppression in adult-thymectomized mice treated with antilymphocyte serum (ALS). A schedule for pulsed antilymphocyte serum therapy to maintain prolonged immunosuppression will be determined.

Major Findings: The contractor has determined optimal conditions for producing a very potent, non-toxic anti-lymphocyte serum (ALS). The methodology for quickly and accurately measuring the potency of each preparation has been defined. In the future, the contractor will prepare quantities of ALS upon demand by program.

Significance to NIAID Program and Biomedical Research: Mycobacterium leprae cannot be grown in vitro, and its growth in vivo is so slow that experiments must be continued many months before an answer can be obtained. An animal model in which the organism would grow rapidly and produce disease similar to that in humans would do much to speed progress in leprosy research.

Proposed Course of Project: The contract will terminate on June 25, 1973.

Date Contract Initiated: June 1970

Current FY Funding Level: $33,477

UNIVERSITY OF HAWAII (NIH-72-2075) . . . Leprosy

Title: Leprosy Surveillance of Pingelapese Population

Project Director: Dr. Robert Worth

Project Officer: Dr. Paul D. Lambert

Objectives: To conduct scheduled examinations of the Pingelapese population in the Ponape District of the Eastern Caroline Islands to determine whether three years of mass chemoprophylaxis with DADDS has broken the leprosy transmission cycle. Annual examinations are planned for 1972, 1973, and 1974. Biennial examinations are planned for 1976, 1978, and 1980.

Major Findings: Conclusions from the results of the 1972 annual examination are as follows:

1) Two or three injections of DADDS does not appear to prevent the onset of leprosy in persons about to develop the disease.

2) Three or four injections of DADDS suppresses the onset of the disease for the duration of the injections and for about 6-12 months thereafter.

3) Three persons developed leprosy after the DADDS injections were discontinued in the general population. These people received less than 10 injections. There have been no cases of leprosy observed in those people who received all 15 injections. From this it would appear that there is a threshold number of injections of DADDS for leprosy prophylaxis.

The 1973 annual examination has been completed, and a report is now being prepared.

Significance to NIAID Program and Biomedical Research: The results of continued surveillance of this unique population, treated prophylactically for leprosy, could have a substantial impact on the treatment and possible eradication of endemic leprosy throughout the world.

Proposed Course of Project: The contract will be reviewed for continued support at the end of the contract year.

Date Contract Initiated: January 1972

Current FY Funding Level: $8,100

Title: Chemotherapy Trials in Leprosy

Project Director: Dr. Edwin M. Lerner

Project Officer: Dr. Paul D. Lambert

Objectives: The Philippine Division of the Leonard Wood Memorial, Cebu City on the grounds of the Eversley Childs Sanitorium, has provided a leprosy research laboratory where clinical trials with drugs of interest can be performed. The Philippine government supports clinical care and management of leprosy patients, whereas studies associated with chemotherapy trials is being supported through research contracts. It is planned that strong bacteriological and pathological backup will continue to be provided by this research facility. Evaluation of several different treatment regimens is presently being provided utilizing the mouse footpad technique, and it is hoped that all animal studies will eventually be conducted at this facility. Because the facility is in a leprosy endemic area, untreated patients are admitted at a rate of approximately 4 per month to chemotherapy trials now being conducted. In addition, through the Philippine Health Department in Manila, skin biopsy material is being provided to U.S. investigators.

Major Findings: Results of the B663 (clofazimine) trial which was completed this year, indicate that the daily regimen is more efficacious than the intermittent regimens. Therefore, since B663 causes an undesirable discoloration in the skin of patients and is most efficacious with daily administration, it is indicated only for DDS resistant cases.

Initiated about 2 years ago was a short-term inpatient trial of acedapsone (DADDS) vs. acedapsone plus intermittent rifampin vs. daily rifampin in lepromatous leprosy. About sixty patients have been recruited to date for this study. As patients complete their assigned regimens, they are placed in a long-term course of the disease. Also, in the long-term treatment trial the efficacy of DADDS treatment will be compared with DDS treatment. Approximately 100 patients will be studied over a course of 5 years.

Initiated this year was an outpatient trial to determine the minimum length of treatment needed to treat paucibacillary leprosy with DADDS alone, and with DADDS plus daily rifampin and DDS. About 40 patients will be recruited and followed for 3 years after clinical and histopathological inactivity has been reached.

Significance to NIAID Program and Biomedical Research: This facility, in an endemic leprosy area, provides an important source of patients available to undergo clinical trials with anti-leprotic drugs.

Proposed Course of Project: This project will be funded as long as there is a need for the services provided.



TIB
EXTRAMURAL
RRB

Date Contract Initiated: March 18, 1969

Current FY Funding Level: $180,000

Title: To supply Thymectomized-irradiated Mice Treated with Bone Marrow to Investigators

Project Director: Dr. Charles C. Congdon

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor provides immunosuppressed adult thymectomized mice to leprosy researchers to investigate the usefulness of this animal model for the growth of M. leprae. In addition, the contractor studies the immune status of the irradiated animal.

Major Findings: The contractor has demonstrated marked defects in the immune response of thymectomized-irradiated mice chimeras. Anatomical studies of lymphatic tissues revealed a gross reduction in size of peripheral lymph nodes at one or two months after thymectomy. The germinal centers were almost completely absent in experimental mice at 1 month but were present in numbers equal to controls at 2 months. Lymphocytes were greatly reduced in the so-called thymus dependent areas of the spleen and lymph nodes at 1 and 2 months following treatment. However, some restoration of lymphocytes in the thymus-dependent region of the spleen was noted in the 2 months group. No residual thymus tissue was found in the thymectomized mice at autopsy. Tail skin allografts were rejected more slowly. The mean rejection time was 20 days compared to 10 days in normal control animals. Humoral antibody to rat red blood cells was not produced in significant amounts even after tertiary challenge.

Significance to NIAID Program and Biomedical Research: Mycobacterium leprae cannot be grown in vitro, and its growth in vivo in the mouse footpad model is so slow that many months are needed before evaluation of the assay method can be made. An animal model in which the organism would multiply more rapidly in significant quantities to provide the researcher with live bacilli would do much to speed progress in leprosy research.

Proposed Course of Project: The project will be terminated June 30, 1973.

Date Contract Initiated: July 1970

Current FY Funding Level: $55,000

STANFORD RESEARCH INSTITUTE (NIH-70-2283) . . . Leprosy

Title: Metabolism of Dapsone in Leprosy Patients

Project Director: Dr. John H. Peters

Project Officer: Dr. Paul D. Lambert

Objectives: In recent years leprosy researchers have determined that the principal metabolite of DDS in plasma, MADDS, is enzymatically acetylated by the same system which acetylates INH and sulfonamides. The speed at which a patient acetylates, slow acetylator vs. fast acetylator, in terms of therapeutic response of patients to DDS therapy, is not known at this time. To obtain additional information about this physiological response, leprosy patients in the Philippines, Japan, India and other countries will be classified as to acetylator phenotype using sulfamethazine and DDS. Also, the effect of continued DDS therapy on metabolism of patients at the USPHS hospital in San Francisco will be investigated. Procedures will be initiated to increase the sensitivity of the fluorometric method to the one nanogram level for tissue specimens.

Major Findings: Completed during this year was an assessment of the characteristics of South Indian subjects to acetylate sulfamethazine (SMZ) and dapsone (DDS) and to clear DDS and monoacetyl dapsone (MADDS) from the plasma. In 49 subjects, slow, intermediate and rapid SMZ acetylator phenotypes were found. Tests with DDS in the same subjects showed a similar type of acetylation capacity. The results are similar to those found earlier in a group of 32 Filipino subjects.

In a group of 21 African leprosy patients no intermediate acetylators were found, but the same parallelism of SMZ and DDS acetylation was noted. No relationships between type of disease and acetylator phenotype was found.

New data gathered to assess the therapeutic significance of acetylator phenotype suggest some association between the emergence of M. leprae resistance to DDS and a greater frequency of intermediate and rapid acetylators in the group. A more rapid rate of clearance of DDS and MADDS from the plasma was also related to DDS resistance.

Continuing efforts to simplify and improve chromatographic-fluorometric procedures have led to the ability to measure ng quantities of DDS, MADDS, and DADDS in plasma by a single run, instead of the two previously required. The total analysis time has been reduced by about 50% and the sensitivity increased tenfold, e.g., 0.1 to 0.2 ng per ml of plasma can now be detected.

Significance to NIAID Program and Biomedical Research: Since DDS is still the drug of choice for treating leprosy, it is important to learn as much as possible about the metabolism of this compound within the body. The study is providing useful data for future drug therapy.

Proposed Course of Project: The contract will be reviewed for continued support at the end of the contract year.

Date Contract Initiated: June 1970

Current FY Funding Level: $64,320



TIB
EXTRAMURAL
RKB

STANFORD RESEARCH INSTITUTE (NIH-70-2286) . . . Leprosy

Title: Synthesis and Biological Evaluation of DDS Analogs and Sulfonamides

Project Director: Dr. Joseph I. DeGraw

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor plans to synthesize various simple analogs of dapsone, pteridine conjugates of certain sulfones, dapsone analogs modeled after sulfamethoxine and other compounds of close structural relationship to the sulfone in hopes of finding effective new antileprotic agents. The synthesized compounds will be evaluated *in vivo* and *in vitro*.

Major Findings: Good evidence has been accumulated indicating that sulfonamide and sulfone compounds do act via a related mechanism involving the incorporation of p-aminobenzoic acid (PABA) into dihydrofolate. The addition of either of the latter two compounds reversed the growth inhibitory action of both DDS and sulfamethoxolate in a model system, *Mycobacterium sp.* 607.

The enzyme, dihydrofolate reductase of the *Mycobacterium sp.* 607, represents the second site of sequential blockade of the *de novo* mycobacterial folate pathway. A series of 2, 4-diamino-5-methyl-6-alkylquinazolines were prepared and found to be strong growth inhibitors of *M. sp.* 607, efficient inhibitors of the reductase enzyme, and potentiators of DDS action.

Significance to NIAID Program and Biomedical Research: Dapsone is used world-wide in the treatment of leprosy and is at this time the drug of choice. By synthesizing and studying dapsone analogs and derivatives, an attempt will be made to relate chemical structure with drug activity. This information could ultimately be used to synthesize a more effective drug.

Proposed Course of Project: This contract will not be renewed because of budgetary restrictions. It is hoped that this activity can again be funded in the future.

Date Contract Initiated: June 1970

Current FY Funding Level: $71,606

USPHS HOSPITAL, SAN FRANCISCO (NIH-IAA-9910) . . . Leprosy

Title: Duplicate Mouse Inoculations of Skin Biopsy Specimens

Project Director: Dr. Louis Levy

Project Officer: Dr. Paul D. Lambert

Objectives: To continue to provide back-up testing and evaluation of the mouse footpad technique by performing duplicate mouse innoculations of skin biopsy specimens from leprosy patients in a chemotherapy trial. The specimens are shipped from the Leonard Wood Memorial Hospital in Cebu, Philippines to the PHS Hospital, San Francisco.

Major Findings: To date, there has been good correlation between the mouse footpad testing of the Cebu and San Francisco Laboratories. The monitoring of the San Francisco Laboratories is helping the Cebu Laboratory to gain experience in making the more difficult evaluations of tissues harboring fewer organisms as chemotherapy progresses.

Significance to NIAID Program and Biomedical Research: This project insures the reliability of results from a chemotherapy trial in leprosy patients.

Proposed Course of Project: The project will be funded as long as there is a need for the services provided.

Date Contract Initiated: July 1970

Current FY Funding Level: $28,800

FOOD COMPOSITION TABLE FOR USE IN EAST ASIA . . . . . Malnutrition

Intraagency Agreement - Nutrition Program, HSMHA

Contract - American Institute of Nutrition (NIH 70-2086)

Project Director: Dr. W. T. Wu Leung

Project Officer: Dr. B. T. Burton (NIAMDD)

Objectives: To prepare a table of food composition for foods used in the East Asian countries of Burma, Thailand, Laos, Vietnam, Cambodia, Malaysia, Indonesia, Philippines, Taiwan, Korea and Japan. This includes indigenous food products as well as imported food-stuffs.

Major Findings: Food data are being collected through correspondence and by personal visits to the various countries. The information collected includes data on moisture, minerals, vitamins, amino acids, fatty acids, trace elements, local methods of preparation, processing and food preservation. Whenever feasible, local names used in various Far-Eastern countries with the corresponding English and scientific names are recorded and collected.

Significance to NIAID Program and Biomedical Research: The first Far-Eastern Food Table was issued in 1945 under the auspices of the Committee on International Food Value Programs of the National Research Council, U.S.A. Although it was revised and enlarged in 1952 as Agriculture Handbook No. 34, "Composition of Foods Used in Far Eastern Countries", it is not applicable to conditions in 1972. Comprehensive food tables for local foods in East Asia are essential for and basic to any attempt to improving the nutritional state of East Asian populations through a combination of nutrition education or nutrition improvement through introduction of innovations in the local food consumption.

Proposed Course of Project: The work under the contract has been completed, and the food tables are ready for printing.

Date Contract Initiated: January 29, 1970

Current FY Funding Level: $1,762

HARVARD UNIVERSITY (NIH 68-1512) . . . . . Malnutrition

Title: Study of Malnutrition and Mental Development in Bogota, Colombia

Project Director: Dr. Frederick J. Stare

Project Officer: Dr. Merrill S. Read (NICHD)

Objectives: To study effects of malnutrition on psychological development and mental functioning in children. To conduct a one-year study of sex-matched siblings in the slums of Bogota, Colombia. To analyze data obtained from nutritional, biomedical, psychological and social measurements. To evaluate the success of the nutrition intervention program and the methods of quantifying the data obtained with psychological tests which have been developed.

Major Findings: The contractor attempted to determine whether malnutrition caused mental retardation or whether other environmental factors related to poverty were of equal or greater importance. These studies involved selected pairs grouped into four categories of various degrees of nutrition. All members received a supplemental diet aimed at achieving rapid recovery of the malnourished sibling and the prevention of malnutrition in the well-nourished one. However, it was determined that the study design was not practical because it was impossible to fill some of the study categories; therefore, it will not be done. The psychological tests specifically devised for this study proved to be most useful as did the supplementation methodology. The contractor is preparing a summary of the research findings in regard to the methodology used and the problems encountered in attempting to follow the field study design. The report will eventually be submitted for publication.

Significance to NIAID/NICHD Program and Biomedical Research: This contract represented a major attempt to develop a new study of nutrition and mental development with controls for genetics and the micro-environment of the family.

Proposed Course of Project: The contract will terminate January 15, 1974.

Date Contract Initiated: June 1968

Current FY Funding Level: $75,000

INSTITUTE OF NUTRITION OF CENTRAL AMERICAN AND PANAMA (INCAP)
(NIH NICHD NO1-HD-2-2737) . . . . . Malnutrition

Title: Analysis of Data from an Ecological Study of Infection, Malnutrition and Growth of Children in a Guatemalan Indian Village

Project Director: Dr. Leonardo J. Mata

Project Officer: Dr. Merrill S. Read (NICHD)

Objectives: To analyze and interpret data from a longitudinal study of nutrition, infection and growth among pregnant and lactating women, young children and infants in a small village in Guatemala.

Overall, four study objectives have been identified:

1. Elucidation of the relationships between gestational events and growth and infection in the first year of life.

2. Elucidation of the post-partum relationships between infection, nutrition and growth.

3. Identification of selected viral isolates from fecal samples in storage as needed.

4. Preparation of a monograph which will describe and summarize the collected data as well as present analytic resluts and interpretations.

Major Findings: The outline of the monograph for the presentation of this study has been completed. It will include data accumulated between February 1964 and January 1972. Although the statistical analyses have not been completed, preliminary data concerning prematurity and fetal growth retardation, birth weight, gestational age and survival, birth weight and postnatal growth, immunoglobulins in cord serum in relation to gestational age and birth weight are extremely informative. Screening and identification of virus isolates of the first months of life of cohort children is continuing. The effect of infection on growth etc. will be carefully analyzed. It will take another year before the full impact of these findings may be realized.

Significance to NIAID/NICHD Program and Biomedical Research: The majority of the world's people live under poverty conditions where both malnutrition and frequent illness exist together. It has been suggested that malnutrition and infection have a synergistic deleterious effect on child growth and development. The data to begin answering this question are being analyzed under this contract.

Proposed Course of Project: The analyses of the data should be completed during the next contract year. The contract is due to terminate May 31, 1974.

Date Contract Initiated: April 1, 1972

Current FY Funding Level: $64,500

INTERNATIONAL RICE RESEARCH INSTITUTE (PH-43-67-726) . . . . Malnutrition

Title: Improvement of the Protein Content of Rice

Project Director: Dr. B. Juliano

Project Officer: Dr. B. T. Burton (NIAMDD)

Objectives: To determine the feasibility of incorporating high level protein strains into commercially produced rice in Asia. To determine the amino acid profile of the new rice protein. To determine protein efficiency ratios in rats and to carry out nitrogen balance studies in man.

Major Findings: The International Rice Research Institute rice collection (7500 varieties) has been analyzed for protein content and 100 contained at least 13% as compared to 7% for commercial rice. Selected high protein varieties were crossed with successful ideal commercial varieties and the high protein quality was incorporated permanently into several sequential generations. Breeding experiments are in progress to incorporate disease resistance into the new protein-rich varieties. Encouraging results from the nitrogen balance studies in adults have made it feasible to plan the same type of study in rapidly growing infants and preschool children.

Significance to NIAID Program and Biomedical Research: Ordinary commercial rice does not contain enough protein to support normal growth and development of infants and children maintained on a predominant rice diet, and does not protect the adult population from serious protein under-nutrition. Rice is the principal food of 60 percent of mankind. If the present protein level of rice (approximately 7 1/2 percent) could be elevated by several percentage points, an automatic and sweeping nutritional improvement would take place in all the populations for whom this one crop represents the nutritional mainstay. Such a development would concomitantly relieve the current burden of the United States which supplies hundreds of millions of dollars' worth of supplementary foods to underdeveloped rice-eating countries. The potential nutritional improvement among Asian populations and under-developed rice-eating countries throughout the world is an important aim of the U.S.-Japan Program and also of the applied nutrition program of the NIAMDD which has scientific cognizance over the malnutrition portion of the U.S.-Japan Program.

Proposed Course of Project: During the next contract year, the breeding and production portions of the workscope will be phased-out with continued and increased emphasis on human nitrogen balance studies using some of the new rice strains.

Date Contract Initiated: May 10, 1967

Current FY Funding Level: $40,170

LINICAL PHARMACOLOGY RESEARCH INSTITUTE (NIH-72-2525) . . . Parasitic Diseases

itle: Cultivation of Molluscan Cell Lines

roject Director: Dr. Eder Hansen

roject Officer: Dr. Kenneth Phifer

bjectives: To establish a primary molluscan cell line in which cells multiply nd form a monolayer and to define and describe growth requirements and hand- ing of these cells so that they may be carried with relative ease by workers n other laboratories.

ajor Findings: Aseptic techniques have been developed and suitable media ave been designed. Primary cultures from snail embryos have been established. hese cultures show a low level of cell division. Since they survive for one o two months, there is considerable opportunity for useful studies. The ontractor is currently working with primary molluscan cultures into which chistosome sporocysts isolated from the snail host have been introduced. hese sporocysts have undergone development to cercarial embryos.

ignificance to NIAID Program and Biomedical Research: A strong need exists or a cell line derived from the schistosome intermediate host, the snail. he need for this tool is most keenly felt by parasitologists interested in nail-schistosome interactions and in in vitro culture of the parasites. The stablishment of cell lines would contribute to our knowledge of the snail ost environment and the specific requirements for growth of schistosome arval stages in vitro. Subsequent in vitro production of large numbers of arasites would make possible immunological, physiological and biochemical tudies which are not technically possible at the present time in the in vivo ystem. Such knowledge is fundamental to the ultimate formulation of rational ethods of control.

roposed Course of the Contract: The immediate aims of the project are xpected to be reached in three years at which time, the project will be eviewed regarding further development.

ate Contract Initiated: June 28, 1972

urrent FY Funding: $17,554

UNIVERSITY OF CALIFORNIA, LOS ANGELES (NIH-72-2526) . . . Parasitic Diseases

Title: Cultivation of Molluscan Cell Lines

Project Director: Dr. Marietta Voge

Project Officer: Dr. Kenneth Phifer

Objectives: To establish a primary molluscan cell line in which cells multipl and form a monolayer and to define and describe growth requirements and handling of these cells so that they may be carried with relative ease by workers in other laboratories.

Major Findings: Experiments designed to establish molluscan embryonic cells in vitro have yielded several consistently reproducible results:

1. Attachment of dissociated cells and of embryo fragments.
2. Formation of fibroblasts within a few hours after explanting of cells.
3. Migration of cells from tissue fragments.
4. Maintenance (and some cell multiplication) in primary cultures for several weeks.
5. Transfer of cells from primary cultures to fresh culture vessels and attachment of these cells.

Significance to NIAID Program and Biomedical Research: A strong need exists for a cell line derived from the schistosome intermediate host, the snail. The need for this tool is most keenly felt by parasitologists interested in snail-schistosome interactions and in the in vitro culture of the parasites. The establishment of cell lines would contribute to our knowledge of the snail host environment and the specific requirements for growth of schistosome larval stages in vitro. Subsequent in vitro production of large numbers of parasites would make possible immunological, physiological and biochemical studies which are not technically possible at the present time in the in vivo system. Such knowledge is fundamental to the ultimate formulation of rational methods of control.

Proposed Course of the Contract: The immediate aims of the project are expecte to be reached in three years at which time, the project will be reviewed regarding further development.

Date Contract Initiated: June 30, 1972

Current FY Funding: $18,220

UNIVERSITY OF GEORGIA (NIH-69-92) . . . Parasitic Diseases

Title: Filariasis Repository and Chemotherapy

Project Director: Dr. Paul Thompson

Project Officer: Dr. Kenneth Phifer

Objectives: To maintain colonies and life cycles of vectors and parasites for several species of filariae to be used in medical research; to supply qualified investigators with filariasis research materials in response to authorized requests; to evaluate selected compounds for antifilarial activity in experimental animals.

Major Findings: During the past year 79 requests were filled supplying filariae and vectors to investigators working on the immunology, chemotherapy, metabolism, fine structure and host-parasite relationships of filariae. Forty-nine compounds were evaluated for antifilarial activity. Five of these showed activity against either the microfilariae or adult worms of L. carinii.

Significance to NIAID Program and Biomedical Research: Basic research in filariasis has been slowed by the difficulty inherent in the maintenance of the life cycles of filarial parasites. The supply of filarial material has encouraged immunologists, biochemists, pharmacologists, etc. to undertake needed studies in this area. In addition, there has been a need for an intensified search for better chemotherapeutic agents. The contractor has been able to apply both his expertise and his laboratory facilities to this aspect of filariasis research.

Proposed Course of Project: This project will be funded as long as there is a need for the services provided.

Date Contract Initiated: January 22, 1969

Current FY Funding: $74,015

UNIVERSITY OF MICHIGAN (PH-43-67-736) . . . . Parasitic Diseases

Title: Supplying Animals Infected with Three Human Schistosomes

Project Director: Dr. Elmer Berry

Project Officer: Dr. Kenneth Phifer

Objectives: To maintain colonies and life-cycles to supply parasites, infecte animals and intermediate hosts of the three human schistosome species and other schistosome materials to selected investigators.

Major Findings: During the past year, 207 requests were filled supplying schistosome-infected mammals and snail hosts to 37 investigators. This represents, an increase of 22% over the number of shipments made during the previous year. In addition, the contractor's staff provided short-term training in routine procedures of snail maintenance to investigators involved in schistosomiasis research.

Significance to NIAID Program and Biomedical Research: Because of substantia technical difficulties in maintaining schistosome life cycles in the laborato the supply of this material enables a number of qualified investigators to carry out research in the field of schistosomiasis. If each investigator had to maintain complete life cycles of this parasite, very few laboratories wo be engaged in schistosomiasis research. Since this disease is second in ter of world-wide morbidity, infecting over 200 million people, research stimulat by the availability of schistosome material is definitely of biomedical significance.

Proposed Course of Project: To continue to supply schistosome material to investigators now working in the field and to stimulate additional investigat to enter this area of parasitological research.

Date Contract Initiated: April 12, 1967

Current FY Funding Level: $79,695

OREGON STATE UNIVERSITY (NIH-72-2527) . . . Parasitic Diseases

Title: Cultivation of Molluscan Cell Lines

Project Director: Dr. Christopher Bayne

Project Officer: Dr. Kenneth Phifer

Objectives: To establish a primary molluscan cell line in which cells multiply and form a monolayer and to define and describe growth requirements and handling of these cells so that they may be carried with relative ease by workers in other laboratories.

Major Findings: Diverse tissues were placed in vitro and observed for the abundance of healthy emigrant cells. Gonad and heart tissues were selected as cell sources. A medium has been developed which has supported metabolically active cells for six months. Beta-glucuronidase and hyaluronidase treatment have been shown to markedly enhance cell outgrowth from gonad explants. Time lapse cinephotomicrography is being used in conjunction with phase contrast microscopy to quantify cell divisions in vitro.

Significance to NIAID Program and Biomedical Research: A strong need exists for a cell line derived from the schistosome intermediate host, the snail. The need for this tool is most keenly felt by parasitologists interested in snail-schistosome interactions and in in vitro culture of the parasites. The establishment of cell lines would contribute to our knowledge of the snail host environment and the specific requirements for growth of schistosome larval stages in in vitro. Subsequent in vitro production of large numbers of parasites would make possible immunological, physiological and biomedical studies which are not technically possible at the present time in the in vivo system. Such knowledge is fundamental to the ultimate formulation of rational methods of control.

Proposed Course of the Contract: The immediate aims of the project are expected to be reached in three years at which time, the project will be reviewed regarding further development.

Date Contract Initiated: June 26, 1972

Current FY Funding: $29,216

HAZLETON LABORATORIES, INC. (NIH-72-2521) . . . Tuberculosis

Title: Study of BCG Vaccination by the Aerosol Route of Subhuman Primates (Macaca rhesus)

Project Director: Dr. Robert C. Good

Project Officer: Dr. Paul D. Lambert

Objectives: To determine the dose-response relationship in tuberculosis-free monkeys vaccinated by differenct doses of aerosol BCG and challenged with aerosols of virulent tubercle bacilli.

Major Findings: Rhesus monkeys were vaccinated with several doses of aerosolized BCG and challenged on the ninth postvaccination week by inhalation of Mycobacterim tuberculosis (TMC 107).

After vaccination, no pulmonary infiltrate was seen on roentgenographs, and tuberculin conversions were for the most part slight. Four weeks post-challenge, all control monkeys had converted their tuberculin reactions, and pulmonary infiltrate was evident on all roentgenograms. Some tuberculin reactions were seen in the vaccinated groups, however, only one monkey showed pulmonary involvement. At six weeks postchallenge six vaccinated monkeys showed pulmonary involvement, but this was less severe than that seen in all of the controls. At the sacrifice date, necropsy data will be collected for evaluation of the BCG vaccination dose.

Significance to NIAID Program and Biomedical Research: BCG vaccination is used extensively throughout the world, but there are many who believe that its present usage could be improved. Program has developed a standardized test method for evaluating the efficacy of tuberculosis vaccination procedures in animals. Earlier work with this method indicated that aerosol BCG was efficacious and feasible. The present work will determine a nominal aerosolized dose which is efficacious in experimental monkeys.

Proposed Course of Project: The workscope of this contract will be completed this year.

Date Contract Initiated: June 28, 1972

Current FY Funding Level: -0-

HAZLETON LABORATORIES, INC. (NIH-72-2534) . . . Tuberculosis

Title: Comparison of Physical Methods for Disruption of Tubercle Bacilli.

Project Director: Dr. Robert C. Good

Project Officer: Dr. Paul D. Lambert

Objectives: The objectives are to prepare by specified methods M. tuberculosis $H_{37}Rv$ culture filtrate and baccili. The bacilli are to be disrupted by specified sonication and pressure cell procedures. The culture filtrate and cell extracts will be analyzed chemically, physically, and immunologically.

Major Findings: Four and eight week culture filtrates have been prepared and analyzed for total solids and total nondialyzable solids. Four and eight week cell extracts have been prepared by sonication and pressure cell procedures and total solids have been determined. The materials are being characterized in preparation for future analytical fractionation research.

Significance to NIAID Program and Biomedical Research: Much work has been done in the past to fractionate and identify biologically active antigens of mycobacteria; however, many preparations and procedures were used, and a diversity of results have been reported. The products produced under this contract will be used as standardized starting materials for an antigen fractionation study using sound and sophisticated methods.

Proposed Course of Project: The workscope of the contract will be fulfilled this year.

Date Contract Initiated: June 28, 1972

Current FY Funding Level: -0-

MELOY LABORATORIES, INC. (NIH-72-2538) . . . Tuberculosis

Title: Preparation of Polyvalent Goat Serum

Project Director: Dr. W. Frederick Hymes

Project Officer: Dr. Paul D. Lambert

Objectives: For Program the contractor has prepared highly characterized quantities of anti-culture filtrate antiserum and anti-sonicate cell extract antiserum. The antisera will be stored and distributed by Program as a part of a standardized reference system.

Major Findings: Mature goats were immunized with either $H_{37}Rv$ Culture Filtrate (CF) or $H_{37}Rv$ Cell Extract (CE) of Mycobacterium tuberculosis. Test bleedings were taken routinely after booster injections. Blood collection was determined individually for each animal after the test bleedings were subjected to immunoelectrophoresis analysis and comparison with reference antiserum pool 001. Sample pools of both anti-CE and anti-CF were submitted for testing, and the final pools prepared under the direction of an ad hoc committee. A total of 1500 ml of each pool was aseptically dispensed into approximately 5 ml aliquots; the remaining serum was dispensed into approximately 50 ml quantities. The lots were labelled as goat anti-culture filtrate (CF) lot No. 002 and goat antisonicate cell extract (CE) lot No. 002.

Significance to NIAID Program and Biomedical Research: Program supplies qualified mycobacterial scientists with research quantities of antigen and antisera. Together these products are used as a reference system by which fractions of mycobacteria can be characterized and related. The reference system has been acclaimed by mycobacterial researchers.

Proposed Course of Project: The workscope has been completed and a final report submitted.

Date Contract Initiated: June 39, 1972

Current FY Funding Level: -0-

Title: Serological Identification of "Atypical" Mycobacteria

Project Director: Dr. Werner B. Schaefer

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor will continue to maintain reference cultures and prepare specific antisera to approximately 25 "atypical" mycobacterial serotypes and supply recommended investigators with these reagents upon approval of the Project Officer. He will also continue work on the serological identification of unclassified strains and improvement of pertinent serological techniques. In addition, he will serve as a reference laboratory for the serology of "atypical" mycobacteria, and to assist the Trudeau Institute in the characterization of reference strains which are to be maintained in the Trudeau Culture Collection.

Major Findings: During the year over 400 isolates submitted from various countries throughout the world were serologically identified by the contractor. Four new serotypes were identified. In addition, the contractor supplied type-specific antisera and reference strains to sixteen investigators from ten countries.

Through collaborative studies, the classification of various strains of mycobacteria by serotyping was corroborated by lipid chromatographic patterns, and the relationship of serologic specificity with colonial variation and pathogenicity was studied.

Significance to NIAID Program and Biomedical Research: A high priority has been placed on the maintenance and standardization of mycobacterial species of clinical significance by the U.S. Tuberculosis Panel. The research and reference laboratory of the National Jewish Hospital with "atypical" mycobacteria augments the culture bank and investigations of the Trudeau Institute with "typical" strains of mycobacteria. Reference laboratories provide numerous services and scientific information necessary for improved research.

Proposed Course of Project: This project will be continued as long as there is a need for the services required.

Date Contract Initiated: February 17, 1970

Current FY Funding Level: $37,108

TRUDEAU INSTITUTE, INC. (PH-43-68-640) . . . Tuberculosis

Title: Authentication, Maintenance, and Distribution of Mycobacterial Cultures

Project Director: Dr. George P. Kubica

Project Officer: Dr. Paul D. Lambert

Objectives: To characterize, distribute and maintain "prototype" mycobacterial strains.

Major Findings: One hundred and fifty strains of biomedically important cultures of mycobacteria have been bottled, taxonomically characterized and are being distributed to approved investigators throughout the world. Six additional strains are being characterized for inclusion in the collection. The distribution of the Trudeau Mycobacterial Culture Collection Catalog resulted in a 30% increased demand for cultures solely for mycobacterial research.

The pathogenicity of 34 strains of tubercle bacilli for mice and guinea pigs were studied. The virulence ranking determined by $LD_{50}$ values resulted in trimodal classification.

Information describing the culture collection and the methods of preservation and storage were published. Studies of improving methods for the preservation and storage of mycobacteria are being continued.

Significance to NIAID Program and Biomedical Research: The contractor maintains a highly pedigreed collection of mycobacterial cultures which are available to competent investigators through the U.S.-Japan Program. Reference reagents, such as PPD, antigen and antiserum are reposited at and distributed by the contractor. The use of characterized cultures and standard reference reagents has already done much to develop research comparability from one laboratory to another in the United States and abroad.

Proposed Course of Project: The contract will be continued as long as Program determines there is an established need by competent investigators for highly characterized cultures and for other reference materials, such as PPD and mycobacterial antigen preparations.

Date Contract Initiated: February 12, 1968

Current FY Funding: $60,676

UNIVERSITY OF MICHIGAN SCHOOL OF PUBLIC HEALTH (NIH-71-2204) . . Viral Diseases

Title: Isolation and characterization of protective antigens of rabies virus.

Project Director: Dr. Edward Eckert

Project Officer: Dr. Kenneth Phifer

Objectives: To isolate, concentrate and identify the essential protective antigens of rabies virus in order to obtain a highly refined and more effective post-exposure vaccine for use in man. This involves the isolation and characterization of protein and glycoprotein sub-units of rabies virus. Another objective of this study will be to evaluate the antigenic potency of sub-unit rabies vaccines and the role of the physical state of the antigens and adjuvants on immune response.

Major Findings: Two preparative procedures have been utilized to dissociate rabies virus into its 5-6 protein components. Guanidine-dithiothreitol extraction led to complete disruption of the virus but also to an irreversible conformational change which altered the antigenic determinants. A more promising procedure was that of virus disruption via Triton x-100, a non-ionic detergent. In this case, there was substantial recovery of antigenic activity.

Significance to NIAID Program and Biomedical Research: One of the principal objectives of the U.S.-Japan Program is the encouragement of research on rabies with emphasis on studies leading to an improved post-exposure rabies vaccine. The standard rabies treatment involves a long series of inoculations of vaccines which do not provide reliable protection and which may evoke paralytic accidents. One of the most relevant areas of endeavour is the isolation and identification of the essential protective antigens of rabies virus with the ultimate objective of developing a sub-unit vaccine which produces strong and lasting immunity in a short period of time after a minimum number of inoculations.

Proposed Course of Project: The immediate aims of the project are expected to be reached in two years at which time the project will be reviewed regarding further development.

Date Contract Initiated: June 29, 1971

Current Funding Level: $21,000

SCRIPPS CLINIC AND RESEARCH FOUNDATION (NIH-72-2535) . . . Viral Diseases

Title: The serologic analysis of dengue hemorrhagic shock syndrome

Project Director: Dr. Frank Dixon

Project Officer: Dr. Kenneth Phifer

Objectives: To quantitate immunochemically the plasma enzyme inhibitors. To quantitate the activity of the anaphylatoxin inactivator. To develop a method for the quantitation of C-3a and C-5a utilizing immunochemical assay techniques to determine whether severe shock occurs only when the equilibrium between enzyme activation and serum enzyme inhibitors is grossly disturbed.

Major Findings: Measure complement components with the exception of C-9 were decreased during shock. Results of metabolic studies have lent support to the concept that activation of complement constitutes an essential part of the pathogenic mechanism of dengue shock. In experimental animals, it has been shown that dengue virus can replicate in primed leucocytes.

Significance to NIAID Program and Biomedical Research: The mechanism by which dengue hemorrhagic shock syndrome develops is unknown. Assessment of enzyme control mechanisms may explain why shock occurs in some patients and not in others. Elucidation of the early events occuring in shock syndrome might yie clues to rational therapy.

Proposed Course of Project: The contract has been terminated

Date Contract Initiated: June, 1972

Current FY Funding Level: None

STATE OF MICHIGAN DEPARTMENT OF PUBLIC HEALTH (NIH-71-2199) . . Viral Diseases

Title: Work on an improved method for inactivation of rabies virus without significant loss of immunogenicity.

Project Director: Dr. John Mitchell

Project Officer: Dr. Kenneth Phifer

Objectives: To develop a safe and effective procedure for the inactivation of rabies virus. The inactivating agent, beta-propriolactone, will be compared with formalin in determining the kinetics of rabies virus inactivation and the effect of the agent on rabies virus antigen. To test for completeness of inactivation and residual levels of inactivating agents.

Major Findings: Hydrolysis of beta-propriolactone (BPL) is not particularly pH dependent at or below pH 8.0, but is strongly dependent on temperature. Rabies virus of a titer below $10^5$ $LD_{50}$ per 0.03 ml. can be completely inactivated with 0.025% BPL at or near pH 7.0. Time and temperature are interdependent. Best protection of the antogen is achieved at 4°c.

Significance to NIAID Program and Biomedical Research: Current rabies vaccines do not produce reliable protection and often evoke paralytic accidents. Thus, strong and lasting immunity in a short period of time with few inoculations. One aspect of the development of an improved vaccine involves the improvement of methods of inactivation of rabies virus without a loss of immunogenicity.

Proposed Course of Project: The aims of the project are expected to be attained within two years at which time, the project will be reviewed regarding further development.

Date Contract Initiated: June 29, 1971

Current FY Funding Level: $43,200

THE WISTAR INSTITUTE (NIH-71-2292) . . . Viral Diseases

Title: Improved methods for potency testing of standard and refined rabies vaccines.

Project Director: Dr. Tadeusz Wiktor

Project Officer: Dr. Kenneth Phifer

Objectives: To develop simple and economical procedures for the evaluation of the protective activity of rabies vaccines by means of an in vivo test in which treatment will follow the virulent virus challenge. To develop in vitro procedures for a rapid determination of the protective activity of such vaccines. To determine which of the presently available serological assays for rabies antibody best measures the state of resistance to rabies infection.

Major Findings: It was demonstrated that interferon induction by inactivated rabies vaccine may play an important role in the mechanism of protection in post-exposure treatment. A radioimmune assay procedure for detecting rabies antigen-binding antibodies was established. Preliminary experiments indicate that in vitro stimulation of lymphocytes can be used to evaluate the potency of rabies virus vaccines.

Significance to NIAID Program and Biomedical Research: The U.S.-Japan Progra considers relevant the encouragement of research on rabies with emphasis on studies leading to an improved post-exposure rabies vaccine since currently available vaccines represent one of the crudest and most unsatisfactory biological products in use in man. One aspect of the development of an improved post-exposure vaccine involves the improvement of methods of potency testing of standard and refined vaccines since existing procedures do not adequately characterize vaccines of tissue culture origin.

Proposed Course of Project: The immediate aims of the project are expecting to be reached in from two to three years at which time the project will be reviewed regarding further development.

Date Contract Initiated: June 1, 1971

Current FY Funding Level: $29,760

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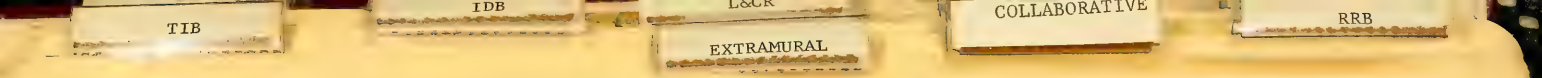
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EXTRAMURAL PROGRAMS - NIAID

TABLE OF CONTENTS


I. REPORT OF THE ASSOCIATE DIRECTOR..... 1

II. PROGRAM REVIEWS..... 8

A. Allergy and Immunology..... 8
B. Bacteriology and Mycology..... 21
C. Biochemistry and Physiology..... 31
D. Parasitology and Medical Entomology..... 36
E. Virology and Rickettsiology..... 41

III. SPECIAL EMPHASIS PROGRAMS (DAPA)..... 47

IV. RESEARCH GRANTS PROGRAM ANALYSIS..... 48

ANNUAL REPORT OF PROGRAM ACTIVITIES

National Institute of Allergy and Infectious Diseases
July 1972 - June 1973

## I. REPORT OF THE ASSOCIATE DIRECTOR

The most unusual feature of Fiscal Year 1973 was the lack of a Congressional Appropriation and the subsequent changes which had to be made without benefit of earlier planning and assessment. An experienced staff and careful management of the budget early in the fiscal year allowed some evenness in awards to research grants applications reviewed later in the fiscal year after our budget was reduced.

The training program phase-out was undertaken with a planned effort to conserve funds which could be used for support of vital research programs and maintain the Institute's momentum in these research areas. Although most training project budgets were reduced, no trainees to whom commitments were made prior to January 29 went unsupported.

The Institute's Extramural Program continued to expand its targeted efforts in asthma and allergic disease centers, venereal disease and important viral infections. Use of the dollar allocation method for supporting Special Emphasis Programs was continued and a method for evaluating Special Emphasis Programs was introduced and evaluated.

### Research Grants

Operations under authorization from the Congressional continuing resolution, in lieu of FY 1973 appropriations, together with reduction of available FY 1973 funds, resulted in appreciably lower funding for competing research grants. It is estimated that about 26 percent of the FY 1973 competing applications reviewed during Council Year 1973 (June 1972, November 1972, and March 1973 Councils) will be awarded (plus 21 FY 1973 requests approved at prior Councils), as compared with 48 percent of the approved grants reviewed for FY 1972. After adjustment was made for approved applications subsequently withdrawn, the figures for percent of approvals funded for FY 1972 and the FY 1973 estimate were 57 percent and 33 percent, respectively. Increase in both direct and indirect costs was reflected in an increase of about 12 percent for the cost of an average grant in comparison to the FY 1972 level. This increase of about 18 percent for the average FY 1973 competing grant related in part to the funding of some additional program project and center awards.

### Training Grants and Fellowships

During the first half of FY 1973 the requirements of the 122 noncompeting training grant applications allowed award of a single new application which was carried over from FY 1972. The latter half of the year was concerned with negotiations to reduce our committed support for ongoing training programs under NIH program phase-out policies. Prior to the decision to terminate

training support commitments had been made to eight competing applications which had received highest recommendations. These applications were supported under NIH policy which allowed support of trainees to whom commitments had been made prior to January 29, 1973. Phase-out operations during the latter half of the year also included termination of operations of the Training Grant Committee.

Of the 37 Research Career Development Award applications approved (69 percent), eight were awarded. For Postdoctoral and Special Fellowships, 28 awards were made from the 174 approved applications. No awards were made from applications reviewed at the January 1973 Training Grant Committee meeting and no spring meeting was held. The program is planned for termination within two years and no new applications will be accepted. The NIAID Allergic Disease Academic Award (K-7) which was approved earlier in the fiscal year could not be activated.

Program Balance

An analysis was made of the changes in the number and dollar amounts for research grants in NIAID's four major categorical disease areas during the period from FY 1969 through 1973. Figure 1 shows the trends in terms of dollar amounts awarded annually in these program areas. It should be noted that the total annual NIAID dollar amounts for all four of these areas from FY 1969 through 1973 were: $50.1 million (FY 1969), $47.1 million (FY 1970), $48.7 million (FY 1971), $55.4 million (FY 1972), and $54.7 million (FY 1973).

While the total dollars awarded decreased from FY 1969 to 1970, and from FY 1972 to FY 1973, funds for research grants in the allergy and immunologically mediated diseases area increased each year during the FY 1969-FY 1973 period (from $12.2 to an estimated $19.3 million). Although in FY 1969 allergy and immunologically mediated diseases research accounted for about 24 percent of Extramural research grant funds, by FY 1973 this had increased to about 35 percent. These grants constituted about 27 percent of the total number of NIAID research grants. During this same interval, funds for viral and rickettsial diseases research decreased from about $15.6 million in FY 1969 to an estimated $12.1 million in FY 1973 (a decrease of from 31 percent to 22 percent of total Extramural research grant funds). The dollar amounts for research grants in the bacterial and fungal diseases area tended to be rather stable during this period: $16.8 million in FY 1969, $16.0 million in FY 1970, $15.6 million in FY 1971, $17.5 million in FY 1972, and an estimated $16.3 million in FY 1973. These grants accounted for about 30 percent of the total dollars and 36 percent of the estimated total number of NIAID grants to be funded in FY 1973. The funding for research grants relevant to viral and rickettsial diseases decreased from $15.6 million in FY 1969 (31 percent of total dollars awarded) to $12.8 million in FY 1970, and has stayed close to this dollar level since then, dropping to an estimated level of about $12.1 million for FY 1973 (22 percent of total dollar amount awarded). Research grants in the parasitic and arthropod-borne diseases area increased from $5.6 million in FY 1969 to $7.3 million in FY 1973 (11 to 13 percent of total NIAID research grant dollars). FY 1973 awards are estimated at $6.6 million, about 12 percent of the total dollar amount awarded by NIAID.

The total number of NIAID research grants awarded in FY 1969 in these four disease areas was 1,337, for a dollar total of $50.1 million. The size of the average grant in FY 1969 was $37,500. In FY 1972, 1,158 research grants were awarded for a total of $55.4 million, averaging $47,800. It is estimated that 1,022 research grants will be awarded with FY 1973 funds, for a total of $54.7 million (an average of $53,500 each). It may thus be seen that increases in direct and indirect costs have been such that the average NIAID research grant increased by about 27 percent (from $37,500 to $47,800) from FY 1969 to FY 1972. Furthermore, this trend has continued into FY 1973, with the average grant estimated to reflect an additional 12 percent increase.

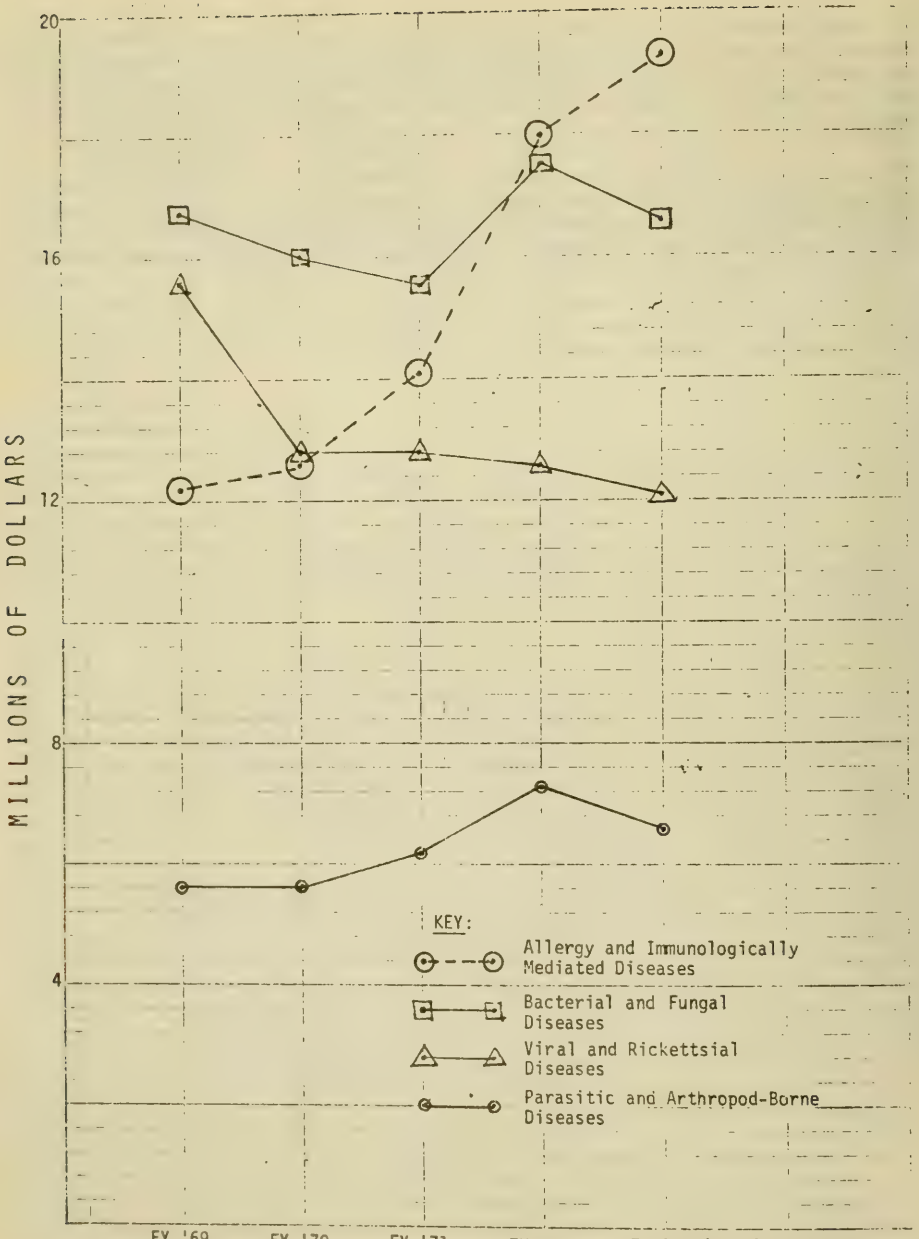


Figure 1. NIAID Research Grant Funds in Four Major Disease Areas (FY 1969-1973)

## Program Evaluation Plan

There has been interest at all levels in the Department in program evaluation. That part of the Extramural Programs which is most readily evaluated is the Special Emphasis Programs. A pilot study of a plan for evaluating these programs was undertaken during FY 1973. Qualitative and quantitative objectives were used in this evaluation plan so that professional judgment was brought to bear on the staff plans for the program, the general performance of grantees whose projects were assigned to this plan and the overall progress which the program was making toward its goals. The professional consultants were ad hoc consultants not associated with the program but familiar with the scientific content. The report from these reviews notes progress, problems, areas that need strengthening, and makes recommendations on changes needed in the immediate future. The report is shared with principal investigators participating in the program and provides a feedback to assist them in further developing their projects toward Institute goals. The Extramural Programs has been pleased with the results of these professional reviews and intends to request funding for their continuation in FY 1974.

## NIH Staff Manpower Study

NIAID's Extramural Program was designated to serve as a prototype for NIH Extramural Programs in an extensive work measurement study which was carried out 1972-1973. This study, which involved the active participation of staff from the Office of the Secretary, as well as NIH's Office of Management Policy and the Office of the Associate Director for Extramural Research and Training, was concerned with measuring the major job functions and work output of NIAID's Extramural Program staff. As part of the study, 14 Extramural Program staff kept daily records of the distribution of their working time spent in various job functions over a four-month period, and results were compared with estimates that had been made previously. Work output measurements that were made included such aspects as grant applications reviewed and grant projects managed. This study, which involved the active cooperation of all NIAID Extramural staff, has as its ultimate goal the development of work measurement supported manpower budgets and ongoing management control systems.

## National Advisory Allergy and Infectious Diseases Council

The Council has been performing at a disadvantage during FY 1973 because of a lack of appointed members. There are currently five vacancies in our Council membership. As routinely scheduled, the terms of three members terminated on September 30, 1972; one member resigned from Council in August in order to accept a staff position; and another member died in November. Nominations for replacement of all of these members have been made. In the case of the three scheduled terminations, the nominations were made by the Institute in April of 1972. Only through the generosity of three former Council members who have been willing to serve as ad hoc members at our meetings has the Council been able to continue its scientific and programmatic responsibilities for review of research grant applications.

Allergy and Immunology Research Committee

This committee was formed primarily to give programs in allergy and immunology which are central to the mission of NIAID but heretofore reviewed by ad hoc peer groups the benefit of continued and comparative assessment by a standing committee of experts in the field. It will also be advisory to the Institute on programs in this area. Its first meeting was held in January, 1973, in which it reviewed 14 Asthma and Allergic Disease Center applications. It also gave primary review to three Allergic Disease Academic Awards, more than 30 fellowship applications and one training grant in allergy and immunology. None of the training programs reviewed was, of course, funded because of the departmental decision to phase out research training programs. The committee held an in-depth discussion on the subject of clinical immunology projects at its May 9 meeting. They also reviewed the content and management of the current NIAID-supported grants in allergy and immunology.

Clinical Consultants

As noted previously, the NIAID, compared to such institutes as NCI and NHLI, has a modest portfolio of clinical programs. This probably reflects the interest on the part of investigators in the etiological agent of infectious diseases rather than the host. In all areas--the allergic, immunological and infectious diseases--the Institute is expanding its effort in clinical investigation. The establishment of the Asthma and Allergic Disease Centers was a pointed effort in this direction.

Two consultants were appointed to make a study of the Institute's clinical programs in allergy and immunology and in virology. Dr. Sheldon Cohen, consultant in allergy and immunology, made a detailed analysis of the training programs in allergy, pointing out that about 20% of the total allergy fellowships and residency programs in the United States and Canada are NIAID-supported. However, the problem is of such magnitude that 18 active training grants in allergy do not begin to supply the manpower needed to deal with the problem. Curtailment of support for research training in the field of allergy and immunology will create special problems for the Institute extramural programs. In FY 1972, there were 112 clinical research grants amounting to about $6.8 million, relating to allergy and clinical immunology. It was of interest that the FY 1972 investment in asthma (where asthma was the subject of primary emphasis) was $580,000, compared to about $1.2 million in transplantation factors.

Dr. Frederick Rasmussen, consultant in virology, made a survey of the status of clinical virology. In his recommendations he was guided by consultations with leaders in the field of virology. A most useful source of information was a panel discussion open to the public held at Atlantic City last September. Dr. Rasmussen's report concluded that (1) knowledge of virus-host cell interactions and pathogenesis of human viral disease are lagging behind contemporary discoveries in fundamental biology. Diseases especially mentioned as presenting the most serious and immediate problems are influenza, diseases caused by herpes viruses, and the collagen diseases. (2) There is a real need for more rapid, more sensitive and less expensive diagnostic

laboratory support for clinical diagnosis of viral diseases. Dr. Rasmussen recommended that special emphasis programs be initiated to attack these two major problems. These recommendations were presented to the March 1973 Council. However, Council deferred action until June to allow their assessment vis-a-vis other NIAID special emphasis programs.

Personnel

During the past year, extramural staff has been most fortunate in both recruitment activities and opportunities for training experiences important to staff development.

In August, Dr. Wilford Bailey, Vice President for Academic Affairs at Auburn University, a former Council member, and former chairman of our Training Grants Committee, and past President of the American Society of Parasitology, joined the staff as Chief of the Parasitology and Entomology Branch. Dr. Sheldon Cohen, a former grantee of the NIAID, a former consultant to our Collaborative program, a well known allergist in Wilkes-Barre, Pennsylvania, and a member of the American Academy of Allergy, joined the staff as Chief of the Allergy and Immunology Branch.

Formal staff training was provided to 24 members of the Extramural Programs, who took 47 courses at a cost for tuition and other expenses of $13,000. This cost did not include their salaries while training; nor did it include the support of the STEP courses, the Grants Associates Program or Upward Mobility Program. Employees who received the longest training programs were Mr. Fred Norton who spent 10 months at Cornell University School of Business and Public Administration, and Dr. Gay, who spent eight weeks at the Federal Executive Institute in Charlottesville, Virginia.

Four employees received quality increases for their sustained superior work performance. They were Lenore Whiton, Anna Mae See, Eleanor Wyatt, and Frances Schlademan.

Dr. Maurice Landy retired as Chief, Allergy and Immunology Branch in March after a distinguished career in government service, including numerous awards recognizing his contributions to the field of immunology.

Mr. R. Donald Reed, who served most recently as Acting Chief, Grants Management and Operations Branch, retired in June, 1973, after completing 39 years of federal service at NIH, which included extensive assignments as a health scientist administrator involved in both the review and the administration of research grants and fellowship awards, in the DRG as well as in EP-NIAID.

Mrs. Virginia Lehr, Supervisory Grants Management Specialist, retired from this key position in the Grants Management and Operations Branch.

Mrs. Eleanor Wyatt has assumed leadership of the entire Grants Management Section, which has been consolidated into a cohesive unit within the Grants Management and Operations Branch.

## II. PROGRAM REVIEWS

### A. ALLERGY AND IMMUNOLOGY

The approximate level of support given allergy and immunology:

| Type of Grant | Number | Amount |
|---|---|---|
| Research grants | 242 | $16,925,000 |
| Training grants | 39 | 2,585,000 |
| Fellowships | 44 | 880,000 |

Summary

The multiplicity of physiologic processes involved in the protection of the body from invasion by microorganisms and foreign protein substances, challenge by incompatible or altered tissue and neoplastic cells, as well as the large number of disease processes that are associated with deviations of the immune mechanism is becoming increasingly apparent. As a result of recent years of highly productive investigation both in basic and clinical areas our expanding fund of scientific information continues to demonstrate that immune mechanisms are involved not only in host defense against infection in the conventional sense but also in tumor growth, aging processes, and hypersensitivity reactions. Diseases of allergy and autoimmunity, host defense processes, transplantation factors, reactions to infectious agents and drug sensitivities all share the common denominator of relating to immunobiologic phenomena.

Especially exciting and important basic areas meriting our encouragement and support have included programs and research dealing with:

- the genetics and biologic processes of the cellular elements of immunity and delayed hypersensitivity (lymphocytes and macrophages);
- origin, chemistry and specificity of antibody classes responsible for effecting humoral immunity and immediate hypersensitivity;
- function of cellular elements (mast cells and basophils) and released chemical mediators in the induction of allergic phenomena;
- enzymatic regulators governing pertinent intracellular functions, i.e., cyclic AMP and GMP;
- genetic and chemical bases for the histocompatibility characteristics of tissues;
- neurochemical beta adrenergic agents affecting cell membrane receptors of allergically involved tissues;
- factors governing immune responsiveness and immune tolerance;

- the antigenic specificity of proteins and carbohydrates endowing plant, animal and bacterial cells, fungus spores, virus particles and pollen grains with the prerequisite immunochemical properties for engendering immune and/or hypersensitivity responses in the challenged host.

It is germane to note that as basic as these investigative problems may appear, research findings have contributed significantly to our accumulating knowledge resulting in many direct and visible applications to the diagnosis and treatment of human disease of atopy, allergy, autoimmunity, and immunodeficiency, surgical transplantation, neoplastic processes, and a variety of internal disorders involving the blood, thyroid, connective tissue of muscles and joints, skin, kidney, lungs and liver.

The productivity, importance and unique relevance of these investigative approaches have justified the continued existence of support for the following special emphasis programs:

1. Asthma and Allergic Disease Centers
2. Clinical Immunology and Immunopathology
3. Lymphocyte Biology
4. Mechanisms of Immunity to Infectious Agents
5. Selective Activation of Cell Mediated vs. Humoral Immunity

Meetings

1. Allergic Disease Centers Workshop: The 2nd Annual meeting for program directors of the 10 Centers and key members of their staffs, organized and sponsored by this Institute, was held in Bethesda on December 4 - 5, 1972. Dr. Richard S. Farr of the Center at the National Jewish Hospital in Denver served as chairman. This took the form of a workshop with discussions largely revolving around attempts to adopt uniform clinical research methodologies and standardized diagnostic procedures in order that collaborative efforts and pooling of data could be effected. Topics included broncho-pulmonary challenge in the differential diagnosis of asthma, cyclic AMP assays, IgE determinations, and skin and mucosal testing with allergenic extracts. An opportunity was also provided for interaction and discussion with invited staff members of the FDA concerning selected new therapeutic agents for the treatment of allergic disorders. The Institute expressed its willingness to cooperate if the program directors desired to hold small interim workshops on selected subjects to enhance collaborative efforts. One such meeting, the Inhalation Challenge Standardization Committee meeting, is scheduled for May 31-June 1, 1973.

2. Cyclic Nucleotides, Immune Responses and Tumor Growth: An NIAID-sponsored (and supported) workshop on the intracellular cyclic AMP system was held January 8 - 10, 1973. Originally organized by Dr. Werner Braun of Rutgers University, the meeting was co-chaired by Dr. Charles Parker of Washington

University and Lawrence Lichtenstein of Johns Hopkins University after Dr. Braun's sudden and untimely death in November. Forty-five investigators representing interacting disciplines in immunology, biochemistry, allergy, pharmacology and cell biology participated. Discussions centered around the relationships of the cyclic AMP system to cellular functions. Intracellular build-ups of cAMP have been associated with quiescence of asthma and allergic states and the inhibition of release of chemical mediators of hypersensitivity. Lowering of cAMP concentrations and possible antagonistic actions of cyclic GMP through reciprocal intracellular levels are associated with adverse effects on these disorders. Other areas of involvement of the cyclic neucleotides were suggested in inflammation, wound healing, tumor growth, phagocytosis and enzymatic destruction of bacteria by blood and tissue cells, cholera and hypersecretory conditions of the respiratory tract. This workshop afforded the participating scientists the opportunity to share their current research data in this rapidly developing and important biomedical area and thus enable them to further and apply their work to the control, treatment and prevention of a variety of disease processes.

3. Mixed Leukocytic Culture Conference: The NIAID contributed support to the MLC workshop held in Madison, Wisconsin, on September 20-22, 1972, through the organization and chairmanship of Dr. Fritz Bach of the University of Wisconsin. Thirty-seven (U. S. and foreign) investigators recognized as international authorities participated in this cooperative approach to discuss a unique laboratory advance in the culture and reactivity of immunologically involved lymphocytes. Their communication enhanced applications of this promising and highly important tool for the evaluation of problems in clinically relevant areas of immunobiology such as transplantation, prevention of tumor growth and cellular immunity.

Publications

1. Genetic Control of Immune Responsiveness. Relationship to Disease Susceptibility, McDevitt, H.O., and Landy, M. This work representing the scientific product of the 5th NIAID-supported Brook Lodge Conference held May 8-10, 1972, was published by Academic Press in November 1972.

2. A compilation of manuscripts representing the proceedings of the Conference on "Cyclic Nucleotides, Immune Responses and Tumor Growth" has been edited for publication and is currently in press.

Asthma and Allergic Disease Centers Program

The thrust of this program continues to be directed to the support of groups with long-term involvement and productivity in laboratory-based immunologic research in areas related to hypersensitivity and the ability to translate their accomplishments to clinical investigations of allergic diseases of man. The program continues to attract energetic and highly qualified investigators representing related disciplines for whom collaboration and correlation of biomedical endeavors in favorable settings and research-clinical environments would be enhanced by provisions of this program. The number of Asthma and Allergic Disease Centers has been expanded to 14; four of these funded in FY

1973. Requests for supplemental funding to expand the capabilities of several centers are being prepared for review.

Program Highlights

AI-10356-03 - K. F. Austen

Some of the mechanisms of the immunologic release of chemical mediators of immediate and delayed hypersensitivity, on the plasma proteins constituting the classical and alternate systems pathways and the interrelationships of blood clotting, lysis of fibrin and generations of the inflammatory producing kinins in allergic disease have been clarified. The pharmacologic activity and chemistry of human slow-reacting substance of anaphylaxis (SRS-A) has been further characterized. Methodology has been developed to measure and control critical steps in these effector systems of immunologic inflammation for their evaluation in inborn and acquired disease; e.g., asthma, hereditary angioedema, systemic lupus erythematosus, cold urticaria, atopic dermatitis and cutaneous vasculitis.

AI-10398-03 - R. S. Farr

A multidisciplinary approach has been directed to an understanding of a heterogeneous group of patients with asthma. Their common denominator has been hyperreactivity to methacholine challenge. However, delineation into more precise etiologic factors has been shown to depend upon IgE-mediated mechanisms, nonspecific irritant air pollutants, and psychological stimuli reaching the bronchi via the vagal nerves. Immune reactions to bacterial products may sometimes trigger bronchospasm associated with bronchitis. Methods have been developed for detecting circulating antigen-antibody complexes related to DNA, as in systemic lupus erythematosus, to investigate their possible roles in some cases of non-reagin mediated asthma.

AI-10304-03 - P. S. Norman

By the use of specially prepared "allergoid" ragweed extracts a dose-response relationship in the effectiveness of immunotherapy in hay fever appears to be emerging. In the study of cAMP levels of lymphocytes of asthmatic patients no abnormalities in the beta receptor activity was noted but indications attributing a lack of cellular response related to calcium levels was suggested. Repeated respiratory challenges of ragweed allergic asthmatic patients with pollen extracts have shown no evidence of priming or desensitization and offers a valid model for the study of drug and immunotherapy effects.

AI-10386-03 - J. H. Vaughan

Well controlled challenge tests have demonstrated the possibility of detecting aspirin sensitivities by pulmonary function abnormalities. A study of bronchial aspirates for bacterial, viral and fungal flora suggests that if microbial factors are responsible for asthma in some patients it is not on the basis of overt infection in the bronchial tree. Histamine-sensitizing factor from Bordetella pertussis has been purified allowing for possible applications in the study of asthma. The diagnostic value of methacholine inhalation

challenge is further evidenced by demonstration of its reliability in discriminating between asthmatics and non-asthmatics.

AI-04646-03 - C. W. Parker

Highly sophisticated immunochemical assays have been developed with important relevance to the diagnosis of human disease states including prostaglandin A, E and F levels in asthma. Studies demonstrating the stimulation of lymphoid cell by haptene-protein conjugates indicated the absolute requirement for macrophages in the primary immune response thus offering leads for the possible value of a test system in the study of immunologic deficiency disease and autoimmunity. Isoproterenol, prostaglandin E, and phytohemagglutin in each activated a separate adenyl cyclase in the cAMP system allowing for highly selective control of different cell functions.

AI-10404-03 - C. E. Reed

In children whose asthmatic attacks were triggered by respiratory tract infections a focus on etiologic factors was possible to investigate. Forty-two percent were associated with cultured viruses of rhinovirus or influenza strains but not parainfluenza, herpes simplex or adenovirus strains or with subclinically shed virus infections. In adults an outbreak of influenza virus-related asthma was identified. Lack of associated bacterial infection and catecholamine discharge suggests appropriate therapeutic approaches.

AI-10400-03 - E. Middleton, Jr.

Experimental data has been gathered to establish correlations between clinical features of asthma and some specific biochemical abnormalities indicating altered adrenergic and cholinergic mechanisms. In asthmatics diminution of intracellular leukocyte adenyl cyclase responsiveness to isoproterenol and increased activity of ATPase with variable reversal by corticosteroid stimulation and prostaglandin E suggests a biochemical heterogeneity in this disease. The favorable action of phentolamine in this system and its blockage by propranolol suggests the possibility of hyperactive alpha adrenergic receptors in asthma.

AI-10391-02 - K. P. Mathews

Instrumentation techniques have been developed for measuring nasal airway resistance (NAR). In a study of patients with rhinitis, allergic subjects tended to respond with the development of increased NAR in contrast to the non-allergic group.

AI-10397-02 - C. E. Arbesman

Sophisticated biochemical techniques have been developed for the fractionation and purification of both pharmacologically active and antigenic components of venoms collected from hymenoptera species. Correlation with clinical studies in man are differentiating allergic from toxic reactions to insect stings. Results are being applied to a study of the indications for and appropriate methods of immunotherapy.

Clinical Immunology and Immunopathology

This program is concerned with the cause, diagnosis, distinctive pathology and treatment of immunologic derangements or abnormalities, transmitted and acquired. In addition to experimental studies, clinical investigations concerned with human diseases arising on immune bases, e.g., immunodeficiencies, autoimmune responses, failure of immune responsiveness, hypersensitivity states have been initiated.

Program Highlights

AI-11313-01 - H. O. McDevitt

This program project at Stanford University is the first NIAID grant support awarded for the establishment of a clinical immunology unit. Its objective is the organization of a collaborating group of highly proficient basic and clinical investigators to deal with the multidisciplinary aspects of immunological diseases. As such they are concerned with applications of research-derived data to the characterization of antigens and autoantibodies in systemic lupus erythematosus, the elucidation of the role of genetic controls of the immune response in autoimmune diseases, and studies of immunocompetent cell populations in autoimmune disorders, leukemia, lymphomas, Hodgkins disease and in patients undergoing immunosuppressive therapy.

AI-07007-08 - F. J. Dixon

This extensive program comprised the work of nine independent workers at Scripps Clinic. Below are selected highlights of that program:

F. J. Dixon: X-ray administration to NZB mice failed to alter the level of viremia in life-long lymphocytic choriomeningitis infections indicating the immunologic regulation was not involved. The development of glomerulonephritis associated with chronic viral infection was related to a multiplicity of different antigen-antibody complexes involving both virus antigens and autoimmune-evoked DNA; mediated by complement the disease can be prevented by anti-complement therapy. The antigen from human glomerular basement membrane involved in nephritis has been isolated.

H. J. Mueller-Eberhardt: Complement activation of platelets was demonstrated to play a role in blood clotting.

J. D. Feldman: Afferent blockage by alloantibody is one mechanism responsible for immunological enhancement for prolongation of skin grafts. Fever in delayed hypersensitivity was demonstrated to arise from a factor released from sensitized lymphocytes in turn generating pyrogen from granular leukocytes.

W. O. Weigel: Thyroditis may be mediated by antibody to thyroid as a result of immunologic unresponsiveness to autologous thyroglobulin at the level of the thymus cell.

C. G. Cochrane: The IgE-basophil mechanism in man was shown to be responsible for the release of a soluble factor causing clumping of platelets and release

of their vasoactive amines responsible for ensuing inflammatory effects on tissue.

R. A. Reisfield: Specific immunoresponsiveness of thymus-derived cells at the time of transplantation is excluded whereas a shift of these cells from the bone marrow to peripheral blood and spleen may be responsible for eliciting a hyperacute graft vs. host reaction.

Lymphocyte Biology

The lymphocyte series of blood leukocytes constitute the critical elements of the immune system through a diversity of highly specialized cellular functions. Individually specific types of lymphocytes are each responsible for recognition of a foreign molecule at its cell surface, transmittal of informational signals to other cells characterized by immunological competence, and production and secretion of specific types of antibody molecules, i.e., immunoglobulins, retention of immunologic memory or the production and release of chemical mediators having biologic activity. This program was therefore developed to attain complete knowledge of the life history of immunocompetent cells and the genetic factors that determine their fate and function. Such knowledge is of great importance in providing information to effectively reconstitute immunodeficient patients, alleviate allergic states, resist virulent infections and to combat neoplastic processes.

To further the advancement of such an approach multidisciplinary programs have been accomplished through NIAID research grants to effectively integrate the techniques of cell culture, cell biology, genetics, virology, immunochemistry and cellular immunology. The two research centers thus far established are at Albert Einstein College of Medicine of Yeshiva University and the Rockefeller University.

Program Highlights

AI-10702-01 - M. D. Scharff

In the initial year of study tumor cell lines synthesizing the major classes of immunoglobulins were characterized and cloned. Techniques were developed to identify variants in order to study the effects of mutagenic agents on the incidence of variants, the genetic and biochemical analysis of variants and the characterization of defects. These have resulted in uncovering lymphocyte cell types resistant to infection with viruses, resistant to killing with antibody directed against their H-2 histocompatibility antigenic components, and blocked in the synthesis or secretion of immunoglobulins. The significance of these findings pertaining to the mechanism of lymphoid cell functions, synthesis of surface cell antigens and immunoglobulins, and activation for replication and differentiation is their application to those relevant aspects of the immune process involved in the rejection of tumors and resistance to infection.

AI-11378-01 - G. M. Edelman

The principal investigator is a Nobel laureate who was awarded the Nobel prize for his studies on the structure of antibodies. He is now also interested in studies on the lymphocyte, in order to attack major unresolved problems in cellular immunology. These investigations form an interrelated program which seeks to correlate lymphocyte functions with the structure of lymphocyte membrane proteins and immunoglobulin products of stimulated lymphocytes as well as to strengthen relationships between lectin receptors, histocompatability antigens and immunoglobulins. Studies underway are directed to the mapping of lymphocyte surfaces for an understanding of cell structure and physiology and to an understanding of the generation of antibody diversity in cellular and genetic terms and the mechanisms of triggering lymphocytes by antigen.

Cell-Mediated Versus Humoral Antibody Responses

This program seeks to understand interrelationships between cell-mediated immunity and humoral antibody formations at the interface of their respective generation, regulation and expression. The capability to selectively manipulate the pathway of the immune response could be applied to the prevention and treatment of infectious diseases and neoplasias and the control of adverse transplantation reactions and aging.

Program Highlights

AI-02154-17 - S. H. Lawrence

Transfer factor (TF) elaborated by lymphocytes has emerged as the most potent immunologic reagent currently available for the induction and reconstitution of delayed type hypersensitivity and cellular immunity in humans. Thus, in immunodeficiency diseases it can confer immunity against life-threatening infections and some tumors. Continuation of these pioneering studies is now showing promise in the development of a precise assay system for TF prepared in the laboratory by lymphocyte culture. It has also been shown that the production of lymphocyte-transforming factor can be dissociated from lymphocyte proliferation and the production of other mediators of cellular immunity also associated with antigen stimulation.

AI-07118-08 - B. W. Bloom

By radioactive labeling and novel techniques for biochemical separation of proteins secreted by stimulated lymphocytes, at least 14 products either newly synthesized or in increased amounts were identified. In a study utilizing pertussis vaccine and BCG to enhance antibody production in spleen cultures, it was found that an induced T-cell released soluble mediator was responsible for the mechanism of adjuvanicity in immunization. It was also demonstrated that by the use of inhibitors of cell mitosis another lymphocyte soluble product of antigen stimulation, the migratory inhibition factor, can be produced by a nondividing (differentiated terminal) T-lymphocyte.

AI-09920-03 - B. Benacerraf

These studies were concerned with the phenomenon of cooperation between specific thymus-derived and bone marrow-derived cells in the immune response and the genetic identification of the responsible specific genes and elucidation of the processes which they control in both cellular and humoral responses to defined antigens. The ability of inbred genetically identical animals to respond with manifestations of both limbs of the immune process was controlled by distinct autosomal dominance. The identified genes were not inherited independently and were found on the same chromosome. A specific immune response gene, controlling responsiveness to a "simple" antigen, can determine specificity for both cellular and humoral immune responses to a more complex antigen.

AI-10280-03 - C. S. Henny

Studies have elucidated the mechanism of target cell destruction by sensitized T-lymphocytes differentiating its pathway from that of B-lymphocyte effects on red blood cells. Agents which can inhibit this effect (e.g., cytochalasin) do so by preventing meaningful association between the lymphocyte and target cell. This cell destruction is mediated by a synthesized protein secreted on the cell membrane and is inhibited by colchicine and cAMP agents. This marked action by cholera toxin on both cell-mediated and humoral immunity may prolong skin graft survivals.

Mechanism of Immunity to Infectious Agents

For the control of infectious diseases, our best hopes lie in an understanding of mechanisms augmenting the body's own natural defenses against microbial invasion and identification of factors, natural and induced, that can favorably influence recovery. Accordingly, an objective of this program is to apply and utilize recent development, research findings, and newly acquired knowledge in immunology to the study of models of infectious disease.

Program Highlights

AI-07015-08 - G. B. MacKaness

The mechanism of immunity studied in models of intracellular infections caused by Mycobacterium tuberculosis and Listeria monocytogenes indicates that (multiclonal) responses against multiple fractions of microorganisms is associated with recovery. Responsible powerful sustained T-cell responses were obtained by priming lymphoid tissues with BCG vaccine leading to resistance against Listeria challenges and these BCG-immune lymphocytes can transfer resistance to normal recipients. There is a gradual transfer of protective activity from the population of young rapidly dividing cells susceptible to destruction to those of the mature long lived types.

AI-10921-01 - J. R. David

A factor secreted by stimulated lymphocytes that will reduce the activity of macrophage cells was identified as identical to one that inhibits their

migration (MIF). Drugs which affect intracellular levels of cyclic AMP in macrophages in turn can modulate the biologic activity of MIF. Macrophages activated by lymphocyte mediators were shown to have enhanced inhibitory effects on bacterial propagation. Prostaglandins may be involved in the activation of the responsible macrophage enzymes. Previous findings were successfully applied to human blood monocytes allowing for evaluation of their cellular functions in human disease. The conversion to positive skin test reactivity in patients with chronic mucocutaneous candidiasis following administration of transfer factor can not be used as a measure of clinical improvement in all cases. Antifungal drugs and transfer factor combined is the treatment of choice.

AI-10295-02 - R. H. Waldman

Induction of respiratory tract and systemic cell-mediated immunity (CMI) were found to be independent phenomena. Respiratory tract CMI and antibody were both best stimulated by local immunization, e.g., inhalation or intranasal application. Their emergence and evolution, however, followed different courses. Local rather than systemic immunization, such as with influenza vaccine, would be most efficacious for induction of immunity in the lower respiratory tract regardless of blood antibody levels achieved by other routes.

AI-10033-03 - C. C. Clawson

The role of human platelets in counteracting bacterial invasion of the bloodstream has been defined. The localization of bacteria deep within large platelets aggregates may protect them from phagocytic processes. Thus when platelets have been made hyper-reactive by exposure to endotoxin, the formation of large aggregates might hinder the protective function of monocytes. On the other hand, platelet-bacterial interaction can be beneficial to the host by localizing bacteria to sites where phagocytosis and chemotactic influences occur.

Other Research Highlights

AI-09273-04 - G. M. Edelman

The elegant work of this investigator in elucidating the structure of antibodies has been recognized internationally by his receiving the Nobel Prize for Medicine for 1972. He has continued sophisticated studies designed to understand the relationship between the structure and specificity of antibodies, the genetic origin of antibody diversity and the connection of antibody structure with fundamental events in cellular immunology, particularly clonal selection. Much of the work involves detailed analysis of the structure of proteins and the development of a method for cell fractionation on surfaces according to their cell membrane receptor properties. These studies have given insights into cellular recognition in the immune response, the general problems of cellular and molecular fractionation and the effect of plant lectins on cell membranes and mitogenesis.

AI-08034-05 - J. W. Uhr

The localization of Immunoglobulin M was identified outside of or attached to plasma cell membrane. In the case of Immunoglobulin G, sites of synthesis and

intracellular transport were also identified by electron microscopic radioautography. Studies to explain the regulation of antibody formation by serum antibodies suggest that there is an equilibrium between antibody in the circulation and antibody in the form of immune complexes in an extracirculatory immunogen-containing compartment.

AI-09652-04 - J. J. Cebra

These studies have elucidated the processes whereby secretory IgA is synthesized, assembled, and passed into the secretions of glandular ducts or intestinal lumen. A majority of lymphocytes in Peyer's patches do contain IgA on their membranes and may serve as a rich source of precursors for secretory antibody plasma cells. Antigenic stimulation of these Peyer's patches cells may result in the migration of primed lymphocytes to the mesenteric lymph nodes and eventually to various secretory tissues where IgA antibody produced by their descendants play a role in infection and immunity at the intestinal lining.

AI-09145-12 - H. H. Fudenberg

These studies were concerned with elucidating genetic mechanisms in immunologic deficiency states. Regulatory defects were identified in several syndromes. Progress was made in the preparation of transfer factor from immunocompetent lymphocytes used in the treatment of Wiskott-Aldrich syndrome to induce cellular immunity. A useful test to predict the results of transfer factor therapy in this condition based upon the identification of defective IgG monocyte receptors was developed. Studies based on the detection of anti-IgM antibodies formed the basis for documenting additional types of immunologic clinical abnormalities. IgM markers in Waldenstrom's macroglobulinemia associated with lymphoma and malignancy were described.

AI-10704-13 - R. A. Good

Important contributions were made to an understanding of the pathogenesis of various forms of kidney disease that affect children. Approaches included both clinical investigation and pathologic studies by electromicroscopy and the study of animal models of induced renal disorders. The role of activation of complement pathways in glomerulonephritis were delineated, including the demonstration of properdin involvement.

In the study of idiopathic nephrotic syndrome, the importance of serum or extrarenal factors and unilateral renal vein thrombosis were demonstrated. In rejection of transplanted kidneys the demonstrated association of absent fibrinolytic activity may offer a new lead for fruitful investigation. Other reported findings of unusual interest relate to the mechanism of entrapment of Immunoglobulin M in kidneys of diabetic patients, the association of recurrent respiratory infection and complement activation with hematuria and focal nephritis, and the absence of glomerular basement antibodies in antilymphocyte serum prepared from lymphoblasts, thus potentially bypassing a complication of ALS treatment in the management of patients with kidney transplants.

AI-10060-03 - K. Ishizaka

This investigator who is responsible for the detection and isolation of IgE continues to extend his work in the study of allergic disease. Pertinent information on the cellular and molecular basis of immediate type hypersensitivity reactions mediated by reaginic antibodies (IgE) is reported. Basophil and mast cell-bound IgE reacting with allergen rather than serum IgE is responsible for allergic manifestations. The number of IgE molecules on the cell surface, enumerated at 10,000-50,000, were less than potential binding sites calculated to be 30,000-100,000. Basophils that are so-called "nonacceptors" are actually acceptors of IgE antibody but respond poorly to antigen-antibody challenge and therefore fail to release histamine. Increased symptoms in patients with pollen-induced allergic rhinitis are related not only to increased local antibody production in the nasal mucosa but also to increased mast cell sensitivity. Immunotherapy may reverse both phenomena.

AI-09565-04 - D. G. Marsh

Studies have been directed to elucidating a genetic basis for atopic allergy in man. From studies of large populations it was found that in highly ragweed-allergic, antigen E-sensitive individuals immune responsiveness manifested by IgE and IgG to a minor allergen (Ra5) is closely associated with the possession of a major histocompatibility antigen of the HL-Al group. The ability to synthesize IgE is inherited as a recessive trait and the control for this antibody level modulates the manifestation of specific immune response (Ir) gene effects.

AI-09268-05 - B. A. Rowley

In the search for ways of specifically suppressing immune responses offering leads for manipulating reactions two new developments have been reported. Specific suppression of antibody response was achieved using homologous antibodies directed against receptors for a specific determinant. An antibody directed against the specific combining region of an antibody was also directed against the similarly structured combining region of a cell receptor for antigen. Thus the anti-antibody raised could either block the activity of antibody already produced or prevent cells from responding initially. Whereas antibody raised against donor antigens caused acute rejection of kidney transplants, pepsin digests of the antibody to give $(FAB)_2$ prevented rejection caused by IgG. Survival of renal allografts in animal models was substantially prolonged by using a combination of the rabbit $(FAB)_2$ and IgG antibodies directed against the donor antigen.

AI-10155-12, AI-09651-02 - P. A. Ward

An inhibitor of leukocyte chemotactic factors was isolated from the pseudoglobulin fraction of normal human serum capable of inactivating each of the three complement-derived chemotactic factors (C3a, C5a, $\overline{C567}$). It is likely that the material in normal serum inhibiting chemotactic factors represents a naturally occurring homeostatic agent and that it functions to provide control of inflammatory reactions. The hemosporidian infection babesiosis

in the rat and mouse has been used as a model to study immunopathologic complications of protozoan infections. Acute glomerulonephritis appearing with but persisting beyond the clearance of intracellular parasitemia has been shown to be an immune complex disorder. Complement has been demonstrated to facilitate the development of parasitemia. While removing the facilitating factors (C3 and C4) limits the extent of parasitized erthyrocytes there was no inhibition to the development of full immunity. The associated complication of anemia has also been shown to be mediated by the presence of complement (C3) and a parasitic antigen on the surface of both parasitized and nonparasitized red blood cells.

AI-08897-05 - D. B. Amos

Macrophages appearing in the peritoneal fluid induced by transplanted ascites tumors are capable of transferring adoptive immunity. It has now been shown that a particular type of lymphoid cell associated with these is capable of cell-mediated cytotoxicity. These effector cells, killed repeatedly, are not consumed during killing, and killing depends upon the number of cell interactions. This model is offering leads to determine exactly how effector cells bring about destruction of the target. Other work has been centered on the study of early events in the induction of immunologic unresponsiveness. Studies on thoracic duct lymphocytes suggest that if tolerance is an active process involving DNA or RNA synthesis, the events that occur in these lymphocytes are not sufficient for replication of viruses.

AI-04967-09 - E. Haber

These studies have been directed to the isolation and examination of structure of homogenous induced antibodies. The completion of amino acid sequencing of light and heavy chains and the location of the third intrachain disulfide bond connecting the available and constant regions of the light chain constitute major accomplishments. It has further been demonstrated that the variable region of the light polypeptide chain comprising 109 amino acids may be cleaved from the heavy chain with retention of its ultimate capacity to reconstitute a combining site. Thus studies leading to the chemical synthesis of the antibody chain have become feasible and are underway. The significant potential application of this is the synthesizing of human antibodies as therapeutic agents in infectious diseases, reversal of toxic drug effects and enhancement of transplanted tissues.

## B. BACTERIOLOGY AND MYCOLOGY

The approximate level of research support in bacterial and mycotic diseases:

| Type of Grant | Number | Amount |
|---|---|---|
| Research grants | 221 | $9,845,000 |
| Training grants | 37 | 2,555,000 |
| Fellowships | 34 | 620,000 |

### Summary

Support of an active and diversified program of high quality research grants is one of the major functions of the Bacteriology and Mycology Branch. Research progress in many of these has been noteworthy, with strong implications for direct application to clinical and therapeutic use. In others, research is uncovering some of the fundamental mechanisms which will be of great future value in helping to unravel some of the unknown areas in the pathogenesis of bacterial and mycotic disease.

The Special Emphasis programs of the BM Branch have continued to stimulate the needed interest of the scientific community and have resulted in many new and innovative research grant applications. Only the best of these grants, however, have been selected for funding under the Dollar Allocation by Program Area (DAPA) system adopted earlier. The Special Emphasis program in Streptococcal Diseases and Sequelae now comprises some 25 grants with total funding of about $1.2 million. No DAPA funds were allocated to this program during the past fiscal year. The Special Emphasis program in Mechanisms of Drug Resistance now has 34 grants, with funding at about $1.25 million. The newest Special Emphasis program, in Basic Biology of Venereal Diseases, comprises 17 grants with funding at $900 thousand. Both of these Special Emphasis programs have received DAPA funds during the past fiscal year. The awareness in the scientific community of the problem areas in venereal disease in further need of research is indicated by the large number of proposals continuing to be received for review.

Other areas of research in the microbiology of important diseases, not covered by the Special Emphasis program, are still of great interest to the biomedical community. Tuberculosis, leprosy, diphtheria, staphylococcal infections, bacterial endotoxins, and the entire broad field of fungal and mycotic infection, continue to be of widespread interest.

A workshop on Hospital-Associated Infections (HAI), organized by the BM Branch and supported by the NIAID, was held in early December 1972. This very grave national problem was discussed in depth by experts in infectious diseases, pediatrics, virology, and epidemiology. A tentative program in which the NIAID could initiate action has been presented to the National Advisory Allergy and Infectious Diseases Council for consideration; the summary of this workshop will be published in the Journal of Infectious Diseases in 1973. The proceedings of the workshop on Mycoplasmatales as Agents of Disease, held in 1970, was published as a supplemental issue to the Journal of Infectious Diseases in March 1973.

The Special Emphasis program on Streptococcal Diseases and Sequelae will be evaluated in April 1973 by a selected panel of experts, under the direction of the Associate Director for Extramural Programs and the associated staff. This program evaluation, upon its completion, will be presented to the Advisory Council at its meeting in June 1973.

## Research Grant Highlights

Some important research findings by grantees of the BM Branch are described here:

### Streptococcal Diseases and Sequelae

#### AI-09527-08 - L. W. Wannamaker

It is a matter of some concern to clinicians that more pneumonitis of streptococcal origin is now being observed, particularly among younger patients. Dr. Wannamaker has found that certain strains of streptococci occur almost exclusively in the upper respiratory tract (URT). These strains are apparently tissue-specific and do not cause later renal or rheumatic disease. The reasons for this local specificity are not fully understood. Other strains of streptococci, which cause impetigo (a skin infection), are also known to cause renal disease (nephritogenic streptococcal strains). These strains were found primarily on the skin, and occasionally in the URT as well. They were not, however, involved in streptococcal pneumonitis. In order to reduce the incidence of impetigo caused by the nephritogenic streptococci, and the subsequent development of renal disease, prophylactic injections of "bicillin" were given. This reduced the incidence of impetigo in a highly endemic area (an Indian reservation) by 38%.

#### AI-09645-04 - E. M. Ayoub

Rheumatic heart disease, with all its debilitating consequences, is essentially a preventable disease. Reliance on primary prophylaxis by the treatment of patients with streptococcal pharyngitis has been of limited value. Understanding the pathogenetic mechanism of rheumatic fever could provide the basis for more rational methods of prevention of this disease.

Dr. Ayoub has found that in patients with rheumatic fever a prolonged persistence of elevated antibody levels to Group A streptococcal carbohydrates occurs. Patients with non-rheumatic myocarditis had normal A-antibody levels during the acute and chronic states of their disease. He has also found that antibody to mitral valve tissue in an animal model system inhibits the uptake of labeled amino acids. Antibody to Group A streptococci also produced some inhibition of uptake of labeled amino acids by human valve tissue. The effect on prevention of myocarditis lesions is unknown.

### Mechanisms of Drug Resistance; Antibiotics

Increasing evidence indicates that R-factors are a major basis for the antibiotic resistance of enteric bacilli producing human disease. These bacilli play an increasingly important role in the etiology of bacterial disease among

hospitalized patients. It is, therefore, imperative that the origin, biochemical basis and regulation of these resistance factors be determined.

AI-08362-06 - D. H. Smith

This investigator has found that an enzyme in E. coli, responsible for chloramphenicol resistance (CAT), is regulated by catabolite repression. It was further found that a different enzyme, beta-lactamase, which inactivates antibiotics with the beta-lactam ring, for example, penicillin, occurred in two separate and distinct entities. The synthesis by the microorganism of these enzymes was also subject to cyclic AMP-mediated catabolite repression. Drug concentrations of gentamycin and kanamycin are normally measured by microbial assays, which are unsatisfactory to determine when blood levels have reached a potentially toxic concentration of these potent drugs in the patient. An enzyme assay has been developed so that rapid and accurate determinations of the blood level of these antibiotics are now possible. This system, with its obvious advantages in therapeutics, is rapidly being adopted by other laboratories as a routine assay method.

AI-00023-24 - L. D. Sabath

Studies have been continued on the biochemical mechanisms of drug resistance, especially on the mechanisms of intrinsic resistance of bacteria to penicillins. Recent results of this study, using radio-labeled penicillin, indicate that in both S. aureus and the gonococcus the surface charges of the microorganisms resistant to the antibiotic were different from those on the organisms sensitive to the antibiotic. This surface charge effect was paralleled by the microorganisms' resistance to penetration and to lysis by bacteriophage. This knowledge could conceivably be of use in therapy of diseases caused by these microorganisms, by using a modified penicillin molecule that could overcome the surface charge effect and penetrate the cell membrane more readily.

AI-09838-03 - J. O. Lampen

Dr. Lampen has been studying the activity of a chemical class of antibiotics called polyenes possessing antifungal activity. Mediocidin, the first hexaene available in the pure form, and a methylester of amphotericin B both showed activity against certain fungal disease agents. The drug's action is directed at the sterols on the cell membrane, which cause a complex formation and therefore alter membrane function of the fungal pathogen. New and modified polyene antibiotics can now be studied using yeast protoplasts as a model system to determine membrane effects.

Basic Biology of Venereal Diseases

AI-10669-01 - R. B. Roberts

Investigations were initiated by this worker to study the interaction between the gonococcus and polymorphonuclear (PMN) leukocytes. He has found that the chick embryo is a useful and reproducible model in which to cultivate the

gonococcus (GC); growth curves showed a 10 to 100-fold multiplication, with a doubling time of one hour. Type 1 (virulent) and Type 3 (avirulent) strains of GC were passed in the chick embryo and both types remained stable after this passage. Data obtained to date indicate that in the presence of fresh, normal human serum the virulent (Type 1) GC resist phagocytosis, whereas the avirulent (Type 3) GC are ingested and rapidly killed by the human PMN leukocytes.

The reasons for this resistance to phagocytosis are not fully understood at present. Elucidation of this resistance mechanism could be of benefit in clinical treatment of GC infections or in prevention of infection by means of serotherapy or specific immune serum fractions.

AI-10615-02 - E. C. Gotschlich

It was previously reported that filamentous structures (pili) were present on the surface of virulent GC cells only. These pili have now been isolated and characterized. Amino acid composition determinations, however, are not yet completed. Purification of the outer membrane of the GC organism also is underway. Antigens from the pili have been used to detect antibody in the sera of infected and asymptomatic carriers of the GC organism. The correlation with female patients and asymptomatic carriers of the GC is excellent. This will form the basis of a rapid and economical serological test to detect the asymptomatic female carrier, one of the major roots of the present epidemiological problem. It has also been determined that females produce from two to three times as much antibody to the GC organism as do males. The reason for this sex differentiation in antibody production is not as yet understood.

AI-10767-02 - S. W. Jackson

These studies have concentrated on the structure, as seen by the electron-microscope, of strains of Treponema pallidum, the causal agent of syphilis. Biochemical evidence provided from these studies strongly indicates that the outer cell envelope of T. pallidum is structurally analogous to the lipo-polysaccharide (LPS) outer membrane of Gram-negative bacilli (GNB). Classical extraction methods were used to obtain the LPS material from the treponemes. Results of these studies lend support to the hypothesized analogy between the treponemal cell envelope and the outer membranes of GNB. If this be true, then this would suggest that an endotoxic activity might be associated with treponemes, and this could play a significant role in the pathogenesis of syphilis.

Cholera and Enteric Bacteriology

AI-07625-07 - C. C. J. Carpenter, Jr.

Two independent groups have confirmed this investigator's observations that cholera enterotoxin directly stimulates adenyl cyclase activity in gut mucosal cells. The toxin stimulation is, however, different from that of naturally occurring hormones; it has a delayed stimulation effect with a lag of three to five hours after exposure of cells to the toxin. The toxin also has a

significant effect on the intestinal crypts themselves. Current approach in chemotherapy is to look for specific adenyl cyclase inhibitors to rapidly reverse gut electrolyte and fluid outpouring caused by the toxin. It was also found that a highly significant, longer lasting protection against cholera, using a dog model system, could be developed when the animals were immunized with purified enterotoxin in Freund's adjuvant. This protection was good for at least 18 months.

AI-11358-01 - R. B. Sack

This investigator found an immunologic similarity between enterotoxins of sero-typically different E. coli, and some immunologic similarity to cholera enterotoxin. Severe diarrheal disease caused by enterotoxin was produced by specific E. coli strains. It was also found that antitoxin antibody in immunized dogs does not protect against an E. coli enterotoxin challenge.

AI-10466-01 - L. J. Berry

It was earlier postulated that the motility of Vibrio cholera contributes to its virulence. It has been found in this study that motile strains of V. cholera were 100% lethal in the mouse model, while non-motile mutants were consistently less lethal. A highly toxigenic cholera strain, with only slight motility, showed a greatly reduced capacity to kill baby mice. Motility also appears to play a role in the attachment of the V. cholera organism to the intestine; it is probably necessary for pathogenesis of the disease. Non-motile vibrios are apparently less able to attach to the intestine.

Dr. Berry is also attempting to develop a new type of cholera vaccine. He has obtained a protein fraction derived from ribosomes of the Ogawa strain of V. cholera. This ribosomal protein material appears to have potential as an immunizing agent against lethal cholera infections, as determined by the results of a study using a mouse-immune model system.

AI-07328-07 - R. Freter

Dr. Freter has been studying the mechanisms which control the normal enteric flora of the gut. He has found that mice, which carried a normal enteric flora, had lower levels of intestinal enzymes and higher levels of intestinal antibody in their serum. This situation did not exist in germ free animals lacking a normal enteric bacterial flora. This is explained by his findings that the normal enterotoxic intestinal flora cause a reduction in levels of proteolytic intestinal enzymes; it is known that these enzymes rapidly destroy serum antibodies of the IgM and IgG classes. Results of this study indicate that the normal bacterial enteric flora control the intestinal antibody levels; these microorganisms at the same time are partly controlled or held in check by these same intestinal antibodies. This feedback system tends to be self-stabilizing in maintenance of the number of the microorganisms in the normal gut.

Studies on Bacterial Toxins

AI-08632-05 - P. F. Bonventre

The mechanism of action of the toxin produced by Cornyebacterium diphtheriae is not fully understood. This investigator found that local infections in guinea pigs with toxin-producing strains of the bacteria caused significant inhibition of protein synthesis in both muscle and non-muscle tissue. The route of diphtheria toxin administration had a profound effect on the pattern of protein inhibition. These findings may have clinical significance when toxin is released from a localized focus of infection in a patient. The data also indicate that the toxin may possess more than one biochemical action mechanism, and that inhibition of protein synthesis may in actual fact not be of critical importance in the pathogenesis of this disease.

AI-08226-06 - S. Osterhout

This investigator has studied the effect of high oxygen pressures on tetanus infections in a mouse model system. Hyperbaric oxygen (HBO) has been used successfully in the treatment of gas gangrene and other anaerobic infections in humans. In the mouse tetanus model, however, it was observed that hyperbaric oxygen showed a decrease in survival time. A single HBO exposure often elicited symptoms of oxygen toxicity and reduced the survival of mice with advanced tetanus. This work suggests that clinical application of HBO for tetanus may be contraindicated.

AI-10784-02 - H. Gewurz

Endotoxin was found to induce the consumption of serum complement in the presence of extremely small concentrations. This complement consumption may be involved in the early phase of shock caused by endotoxemia. An intact coagulation system was found not to be necessary for shock in experimental animals. Methods for control of complement consumption could conceivably have an important effect on control and therapy of shock caused by endotoxemia in infections and septicemia caused by Gram-negative bacilli.

AI-05581-11 - A. H. Nowotny

In studies with endotoxin, it was found that the presence of calcium ions causes polymer-like forms of the glycolipid fraction of endotoxin. The purified glycolipid fraction behaves like an endotoxoid in inducing endotoxin tolerance in guinea pigs; these substances remain in the microcirculation longer than does the unaltered endotoxin. Endotoxin pre-treatment of specific strains of tumor-bearing mice causes the animals to become resistant to tumor induction; this was especially noteworthy after injection by the IP route of the endotoxin. The glycolipid fraction also appears to have ability to induce hemorrhage and necrosis of a specific sarcoma cell in the experimental mice.

Tuberculosis and Leprosy Research

Tuberculosis and leprosy are two different disease entities caused by species of the acid-fast Mycobacteria. Tuberculosis (TB) is still a major health

problem in the United States, with an estimated 40,000 new cases occurring annually. Many of these infections, which do not resemble the classical tuberculosis, are caused by atypical members of the M. tuberculosis group of microorganisms.

It is now well established that a positive tuberculin skin test does not always indicate an infection with the typical TB organism. Other closely related acid-fast microorganisms may cause cross-sensitivity in man. These cross reactions create a steadily increasing problem, as the mycobacteria involved are found in nearly every part of the world. A useful product such as Purified Protein Derivative (PPD), commonly used in TB-screening programs, must be more specific to indicate positive reactions only with the typical tubercle bacillus.

AI-09962-01A1 - G. P. Kubica

Dr. Kubica has prepared large quantities of partially purified protoplasmic extracts from M. tuberculosis for chemical fractionation and purification. These purified protoplasmic products may offer hope for a more sensitive test than the standard PPD material obtained from whole-cell TB organisms. He is also using guinea pigs sensitized to a number of atypical M. tuberculosis. No tuberculin test to date can reliably distinguish infections with TB from those with atypical mycobacteria. The protoplasmic fractions, it is anticipated, will yield a peptide or glycopeptide material suitable as a skin test antigen, thus giving practicing physicians a more specific diagnostic tool in infections with the mycobacteria.

AI-06578-08 - P. T. Davidson

This investigator has continued his studies on the drug therapy of clinical tuberculosis. Drug resistance is a major problem in the treatment of patients. Rifampin is a highly effective anti-TB drug, particularly with drug-resistant mycobacteria. The drug is well tolerated in these patients. Because of its widening use, it is now observed that some patients develop rifampin-resistant TB organisms. This can be avoided by careful evaluation and the judicious use of drugs; some patients can be assisted by a careful study of drug susceptibility patterns. Rifampin is shown to be of value in the treatment of infections with atypical mycobacteria, especially M. kansasii. Successful treatment of atypical mycobacterial infections has been accomplished with five or six drugs in combination, including cycloserine, isoniazid, rifampin and PAS. This offers an approach to the treatment of other atypical mycobacterial infections which have in the past been refractory to treatment.

Leprosy is a serious health problem in many of the underdeveloped nations, particularly in Africa and Asia, with an estimated 15,000,000 lepers worldwide. A small number of cases are found annually in the United States, primarily in those of Mexican, South American or Philippine descent, or in those who have resided in these areas. Current therapy requires long duration of treatment by specific drugs; the development of drug resistance is a major problem in leprosy treatments.

AI-10094-03 - W. E. Bullock

Patients with lepromatous leprosy in this study were given either transfer factor or whole lymphocytes (presumably containing transfer factor) from healthy donors. These patients demonstrated a marked increase in the favorable immune response to their underlying disease, with definite signs of clinical improvement, though of short duration. Chemotherapy with sulfone drugs or rifampin, coupled with repeated doses of transfer factor, could produce a longer immune response, one that would be useful in both primary and maintenance therapy of leprosy. Use of transfer factor in leprosy has created considerable interest, so that a controlled double-blind trial of transfer factor in leprosy treatment is now being planned for the near future.

Studies on Vaccine Development

Newer methods of vaccine preparation are constantly being sought to improve standard vaccines and to develop newer vaccines not now available against specific diseases. One method of promise is the use of vaccines made from the purified ribosomal fraction of bacterial cells. This material hopefully will produce vaccines of enhanced potency and with minimal untoward side reactions in vaccinees.

AI-10449-02 - W. Johnson

Results of this study in purified ribosomal fractions obtained from cells of S. typhimurium indicated that the ribosomes themselves were not immunogenic. However, proteins isolated from purified ribosomes showed strong immunogenic potencies. The data from this work strongly suggests that a ribosomal protein is the most important part of a ribosomal type of vaccine preparation. Ribosomal protein fractions obtained from cells of Streptococcus pyogenes also appear to have immunogenic potency in experimental animals.

AI-01636-16 - G. P. Youmans

This investigator has found that a ribosomal preparation from a mycobacterial strain induces a strong immune response in experimental mice. Further study showed that this highly immunogenic factor resides in a specific double-stranded RNA component of the ribosomal preparation. The mycobacterial RNA was also shown to be a potent adjuvant for induction of antibody in other test systems, such as delayed hypersensitivity studies. The RNA fraction which induces TB immunity, although demonstrated thus far in experimental animals only, could hold out considerable promise for greatly improved human TB immunization.

Fungal and Mycotic Infections

AI-10547-02 - R. I. Lehrer

These studies have succeeded in the functional characterization of an activity within human neutrophils which kills pathogenic yeasts of the Candida species. Results show that a second, myeloperoxidase-independent system in leukocytes is fungicidal, in addition to the lethal fungicidal activity caused by the myeloperoxidase enzyme found in leukocytes. The specific identification and delineation of the leukocyte fungicidal mechanism would have great importance for eventual therapy of Candida infections, especially in immunosuppressed patients.

AI-10622-01 - G. Medoff

The resistance of pathogenic fungi to many antibiotics is based on a failure of the drug to penetrate the cellular membrane of the pathogen. Results of Dr. Medoff's studies show that this can be overcome by using amphotericin B, which directly affects cellular permeability. This has direct clinical implications as an important concept, that cellular integrity of the pathogen can be altered by specific drugs. In the presence of amphotericin B, a specific anti-fungal cytotoxic drug such as 5-fluorocytosine showed greatly increased uptake by pathogenic Candida species, with greatly enhanced killing effects on the pathogen. Other antibiotics can also be potentiated by amphotericin B by increasing their uptake into the cell. This has been shown for rifampicin, tetracycline, and others. Studies with rifampicin were the first demonstration that this antibiotic would inhibit RNA synthesis within fungal cells.

Other Microbiological Research

Pseudomonas aeruginosa, a commonly occurring Gram-negative bacillus found in hospital environments, is the cause of serious infections in the hospital environment. It is also a serious infection commonly occurring in cystic fibrosis patients and in those with burn injuries. Little information is available concerning the mechanism by which this organism causes disease, and the means of control are not satisfactory.

AI-08504-04A1 - P. F. Bartell

Results of this study on the structure and activity of the bacterial slime produced by Pseudomonas aeruginosa indicate that the slime has endotoxic-like properties, which can result in the death of experimental animals. The slime was, in fact, two to three times more toxic than endotoxin of Pseudomonas, and was found to be antigenically distinct from the endotoxin LPS. The slimes were composed primarily of neutral sugar substances, differing somewhat according to the strain of Pseudomonas. The evidence strongly suggests that the slime plays an important role in lethal infections with this group of organisms. Understanding the mechanism of slime formation would be of great value in preventing or treating the serious infections caused by this group of microorganisms.

AI-10334-02 - G. A. Andres

A model system was developed for the study of the immunopathology of renal cortical tubular disease. This was accomplished by the injection of homologous kidney suspensions with Freund's adjuvant together with an injection of pertussis vaccine. Thus it was demonstrated that an autoimmune response could be elicited by antigens of the basement membrane of renal tubules. It was also shown that a comparable mechanism plays a role in the development of chronic interstitial and tubular lesions of human renal allografts. Dr. Andres' studies have also provided additional details concerning the precise site of the antigen-antibody reaction in hyperacute renal rejection phenomenon.

AI-08651-05 - D. Rifkind

Results of these studies with mice immunized with various antigens following treatment with different gonadal steroids have led to the development of a unique hypothesis regarding the role of sex factors in infectious diseases. Dr. Rifkind's working hypothesis is that reactivity of the lymphoid tissues in the intact male is suppressed by gonadal hormones, which are principally androgens. In the intact female, reactivity of lymphoid cells appears unaffected by the gonads. This sex difference in response of lymphoid cells to gonadal hormones could contribute to the increased resistance of the female animal to infectious diseases, but with a greater susceptibility to hypersensitivity (allergic disorders).

C. BIOCHEMISTRY AND PHYSIOLOGY

The approximate level of support given biochemistry and physiology:

| Type of Grant | Number | Amount |
|---|---|---|
| Research grants | 302 | $15,825,000 |
| Training grants | 5 | 403,000 |
| Fellowships | 24 | 465,000 |

Summary

Antiviral substances continues as the single special emphasis program of this Branch. The program has three facets:

(1) Interferon

(2) Bacteriophage

(3) Basic animal virology

No component of the program is accorded a special advantage in the competition for funds.

Much of the recent information and contributions to the understanding of the nature of viruses and the mechanisms of their effects have come from basic research in the areas of the biochemistry, biophysics and genetics of bacteriophage, a bacterial virus. This group of viruses reproduces so rapidly that it provides an excellent research tool for exploring the biochemistry and genetics of viruses in general. The work in this area is extremely important and merits continued strong support.

Although the investigation of the basic cell-virus interaction and viral replication is being pursued actively by research workers in the area of bacterial viruses (bacteriophage), other workers supported by this Institute are studying this phenomenon in animal viruses such as fowlpox, pseudorabies, polyoma, influenza, adenovirus, vaccinia, reovirus, arbovirus, poliomyelitis, rhinovirus, sindbis, and vesicular stomatitis. There is pronounced applicability of findings in bacteriophage to research in animal and human viruses. Our goal in supporting all of this research is a better understanding of the underlying mechanisms of regulation responsible for the replication of viruses. Only when these basic processes are better understood can one logically evolve meaningful antiviral agents with which to attack viral replication at the most vulnerable points.

Except for isolated instances, no generally useful drugs have appeared for the treatment of infections caused by viruses. Drug therapy in viral diseases must await the development of a rational approach to the synthesis of chemotherapeutic agents. This approach can only be fostered by fundamental studies of the virus-cell interaction and through projected basic studies of viral 3-dimensional structure, together with the way in which they grow and replicate. Equipped with this basic virological knowledge,

investigators may then devise means of disrupting the growth of the virus for therapeutic purposes, without injuring the human cell in the process.

RESEARCH GRANT HIGHLIGHTS

AI-05629-10 - T. Merigan

Colds caused by one of the rhinoviruses have been prevented in volunteers by administration of a nasal spray of interferon - a naturally occurring antiviral substance. Although similar experiments, using influenza B virus, were not encouraging, the dose of interferon used in these latter studies was probably too small, according to an NIAID grantee who participated in the research which was carried out in England.

Dr. Thomas C. Merigan, Stanford University, cooperated with investigators at the Medical Research Council's Common Cold Unit, Harvard Hospital, Salisbury, Wiltshire, England in both *in vitro* and *in vivo* studies of interferon prepared outside the body.

Scientists have long hoped to make interferon available as an antiviral drug since it is active against most viruses and has no toxicity. Recently, methods have been developed whereby explanted human cells can be stimulated to produce large amounts of interferon. It was this type of exogenous interferon that was used in Dr. Merigan's studies.

Cell and organ (trachea) cultures were first used to determine the susceptibility of certain respiratory viruses to interferon. Influenza virus and rhinovirus 4 were then chosen for use in the studies involving 54 volunteers.

Interferon given prior to influenza challenge failed to alter the subsequent incidence or severity of infection, although a more detailed analysis of the clinical course of disease in the volunteers indicated that in three of 11 of the interferon-treated group, disease was delayed. This observation was similar to others made in animal infections treated with borderline effective doses of interferon, suggesting that, in future studies, interferon should be administered in larger doses and continued after virus challenge.

In experiments with rhinovirus 4, large doses of interferon were given both before and for three days following challenge with the common cold virus. Only one of the interferon-treated volunteers showed any cold symptoms.

The scientists stress that presently interferon is far from being a practical remedy for the common cold and other respiratory ailments. For example, in the rhinovirus study, it was necessary to give each volunteer the amount of interferon produced by white cells from some 30 to 50 blood transfusions. Dr. Merigan and his co-workers believe, however, that if interferon proves to be clinically applicable, production techniques will be improved, the cost of interferon will decrease, and the method of administration will be simplified.

AI-09921-03 - G. Edelman

This grantee, co-principal investigator on the above grant (together with Dr. George Reeke), was awarded the Nobel Prize in Physiology and Medicine in October, 1972, for his work on the determination of structure of immunoglobulins.

His work on this grant, however, involves concanavalin A, a plant lectin which binds polysaccharides and stimulates lymphocyte transformation. Current interest in this substance centers on demonstrated differences in its effects on binding to various kinds of cells, including normal and tumor cells, implying differences in the surfaces of these cells. Understanding of the molecular basis for the effects of concanavalin A on cells mediated by interaction of concanavalin A with cell surface structures is of great interest in cell biology, and is of particular importance in understanding and exploiting functional differences between normal and tumor cell surfaces.

Knowledge of the complete three-dimensional structure of Con A obtained by Reeke and Edelman makes it at present the best characterized of the various agglutinins and mitogens which are being used in our laboratory and elsewhere to study the nature of cell surfaces and their interactions with specific proteins. It is believed that analysis of the action of Con A provides one of the most promising approaches to understanding the mechanism of lymphocyte triggering in the immune response.

AI-07194-09 - D. R. Helinski

Work has progressed on the genetic and biochemical control of circular DNA elements which will result in an ultimate understanding of a wide variety of essential genetic traits in bacteria and plant and animal cells. This knowledge could also provide some insight to the means of control of the resistance factor, responsible for antibiotic resistance in pathogenic bacteria, tumor-producing viruses possessing a circular DNA genome and aberrant circular DNA forms in the mitochondria of leukocytes of leukemic individuals.

AI-08078-06 - T. Watanabe

This pioneer in the discovery of the resistance factor died during the year. Prior to his death, he was continuing his studies on flavomycin, an antibiotic which has a lethal effect on bacteria containing the resistance factor. His studies on the mechanisms of action underlying the sensitivity of bacteria containing the R factor to flavomycin should, if successful, lead to antibiotics with a greater ability to cope with resistant bacteria. These studies are being continued by his associates.

AI-10096-03 - C. Colby

Dr. Colby is exploring the mechanism of interferon action. Experimental evidence suggests that a product of the cell nucleus is required for the expression of the interferon response. In collaboration with another of our grantees (Dr. Joseph Kates, AI-08413-05), Dr. Colby has found that interferon pretreatment blocks the appearance of vaccinia DNA "factories"

in nucleated cells. However, if they first remove the nuclei and then treat with interferon, subsequent infection with vaccinia virus results in the appearance of a full complement of pox DNA "factories." Thus, the physical presence of the nucleus is required for the establishment of the interferon-induced state. They are currently investigating the time course of this system both from the standpoint of the kinetics of establishment of the antiviral state as well as half-life stability of the state, once established. They are also using autoradiography to visualize the virion-bound transcriptases of vaccinia and vesicular stomatitis virus in enucleated cells. Efforts for the moment are directed toward increasing the sensitivity of the method since the products of the transcriptases are diffusable as opposed to those of vaccinia DNA replicase which are localized. Thus, a definitive analysis of the effect of interferon pretreatment of nucleated and enucleated cells on the virion-bound transcriptases requires quantitation of the numbers of grains in such cells.

AI-05669-10 - E. J. Corey

Fumagillin is an antibiotic isolated from Aspergillus fumigatus. It possesses antiparasitic and carcinolytic activity. The biological activity and novel structure stimulated the interest of several groups in attempts to synthesize the substance. Several factors conspired to defeat the efforts of those interested in synthesizing this complex molecule. Through an approach most appropriately described as elegant, Dr. Corey and co-workers announced the successful synthesis during the year.

Dr. Corey has now turned his attention to another complex synthesis--that of the antibiotic, erythromycin.

AI-10646-02 - P. Sparling

This investigator is studying the resistance of Neisseria gonorrhoeae to antibiotics. His studies indicate convincingly the existence of two separate genetic mechanisms for antibiotic resistance in the gonococcus: (1) independent genes for low-level resistance to each of several drugs, and (2) another gene which results in increased resistance to at least six different drugs. The latter gene may require the presence of the others for its effect to be manifest, suggesting it may regulate the expression or numbers of the genes for low-level resistance.

Extensive studies on the genetics or antibiotic resistance in the gonococcus are now nearly complete. Computer analysis of antibiotic sensitivities of clinically isolated strains had previously shown strong positive correlations in sensitivities to at least five different antibiotics. Studies of mutants to increased sensitivity or increased resistance to several antibiotics had suggested that there were two basic mechanisms for antibiotic resistance: there appeared to be several loci for low-level resistance to each of the antibiotics as well as another single gene which determined higher level of resistance to the entire group of antibiotics. He has now completed genetic transformation studies, in which sensitive gonococci were transformed by DNA extracted from various resistant strains. Dr. Sparling thus obtained better

evidence that there are independent loci for resistance to each of penicillin, tetracycline, erythromycin, chloramphenicol, and streptomycin. The loci for penicillin and erythromycin resistance are not linked to any of the others, but there is close linkage between the three loci for resistance to tetracycline, chloramphenicol and streptomycin. The locus for streptomycin resistance determines high-level stable resistance to this drug; the others determine only very low-level resistance. Most resistant strains also contain another gene which results in maximal resistance to the entire group of drugs: penicillin, tetracycline, erythromycin, chloramphenicol, rifampin and others. This novel gene is only expressed in strains which already have low-level resistance to several of these antibiotics; it is the genetic basis for clinically significant levels of resistance to penicillin. It is possible that the single gene for multiple drug resistance is a regulatory gene, although the biochemistry of antibiotic resistance is still lagging. He has demonstrated with a cell-free protein synthesizing system that high-level streptomycin resistance is a result of mutational alteration of the 30S ribosomal subunit, as expected.

He has also been working for the past year on a bacteriocin typing system which will hopefully be useful in epidemiological studies of gonorrhea. He has now been able to extract in soluble form a high molecular weight, heat-stable, trypsin insensitive, alkali resistant, growth inhibitor which shows some differential activity against different gonococcal strains. There also is some rough variation in the amount of this material which can be extracted from different strains. This material is very tightly associated with the cell envelope and under no condition can be stimulated to be produced into the broth supernatant of a culture. It therefore does not appear to be a true bacteriocin but may be a lipopolysaccharide component of the cell wall. Efforts to develop this system to the point of clinical utility for strain typing are still underway.

AI-09116-05 - V. Najjar

Previous communications from this laboratory have shown that only one particular fraction of freshly prepared human gamma-globulin contains a specific cytophilic gamma-globulin, leucokinin. This binds specifically and reversibly to the autologous polymorphonuclear leucocyte (PMN) and stimulates its phagocytic activity 2 - 2.5 fold.

A study of the mechanism of action of leucokinin indicated that a highly active small fragment was responsible for the full effect of the whole molecule. This phagocytosis-stimulating fragment has been isolated, characterized as a peptide, and its amino acid sequence determined. It has also been synthesized and named tuftsin (after Tufts University).

Two clinical cases, each possessing a deficiency of the phagocytosis-stimulating tetrapeptide tuftsin are described. One is a female whose mother is also deficient. The other is a male whose father is deficient. Each of the children has a history of severe recurrent infections. However, each of the involved parents is without symptoms, the deficiency in the patients was shown to be due probably to a structural defect in tuftsin rather than the complete absence of the tetrapeptide as is most likely the case in splenectomized patients.

D. PARASITOLOGY AND MEDICAL ENTOMOLOGY

The approximate level of support provided for parasitology and medical entomology in FY '73:

| Type of Grant | Number | Amount |
|---|---|---|
| Research grants | 154 | $6,565,000 |
| Training grants | 18 | 1,207,000 |
| Fellowships | 10 | 209,000 |

The research grants were divided among the major groups of parasites as follows:

| Parasite Group | Number | Amount |
|---|---|---|
| Protozoa | 47 | $1,870,000 |
| Helminths | 60 | 2,540,000 |
| Arthropods | 47 | 2,155,000 |

Summary

Research grants in the Special Emphasis Areas constitute approximately 43% of the number and 49% of the funds for the Parasitology and Medical Entomology Program for FY '73. These are distributed between the two areas as follows:

| | | |
|---|---|---|
| Immunity to Animal Parasites | 28 grants | $1,152,000 |
| Biological Regulation of Vectors | 36 grants | 2,090,000 |

The specific diseases supported most heavily by the research grants in this program are: schistosomiasis ($1,073,000), malaria ($737,000), filariasis ($342,000), and leishmaniasis ($306,000). Because of their importance as transmitters of diseases (such as malaria, filariasis and many viral diseases) and as serious pests, more funds (approximately $1,423,000) are being expended this year on research on mosquitoes than on any other group of parasites.

A striking feature of the Parasitology and Medical Entomology Program, as revealed by a detailed analysis of research grants during the year, is the intimate relationship between fundamental studies and "targeted" objectives of prevention, treatment or control of diseases caused or transmitted by a broad spectrum of parasites of man. Several examples will illustrate the intimacy of this relationship:

(A) Parasite Biochemistry Related to Drug Therapy: Very fundamental studies on biochemical metabolism by an investigator supported by NIAID have led to the discovery of some highly unusual enzymes which appear to function in the glycolytic pathway of the disease-producing protozoan, Entamoeba histolytica. That these enzymes are quite different from those operating in the comparable step of metabolism in man sets the stage for directed drug attack aimed at the specific vulnerable points of the parasite metabolism. The mechanisms of these enzyme interactions are being studied now in light of this objective.

Studies in another university supported by the Institute have demonstrated that activity against Schistosoma mansoni is determined by the three-dimensional structure of a drug. This apparently is related to the ability of the drug to block enzyme receptors of the parasite. With this type of information, it has been possible to relate minor structural differences in the chemistry of drugs to the absence of enzyme inhibition in man and thus to the freedom from unfavorable side reactions from the drug. Such knowledge also paves the way for manipulation of the chemical structure in an effort to retain effectiveness against the parasite while eliminating mutagenic or carcinogenic activity.

(B) Mechanism of Immunity in Relation to Vaccination Against Malaria: Basic studies on the mechanisms of immunity against bird and rodent malaria demonstrated that the infective stage of the parasite (the sporozoite, which in nature is transmitted to the host by the mosquito) induces, when inactivated by exposure to X-rays, a considerable and frequently total resistance against infection. Fundamental studies continue to clarify the roles of humoral antibody and T cells and the immunological characteristics of different developmental stages of sporozoites. At the same time, practical studies have proceeded on methods of mass isolation and purification of sporozoites; and immunization experiments have been initiated with X-irradiated sporozoites in Rhesus monkeys. If vaccination is successful in the simian experiments, this spectrum of fundamental studies--many of them on the rodent malaria model--could possibly lead to effective vaccines against malaria in man.

(C) Biological Control of Mosquitoes or Diseases Transmitted by Them: Exhaustive fundamental research by an investigator supported by the Institute has produced extensive information about the biology of Aedes aegypti, including the recent demonstration that susceptibility of this mosquito to bird malaria is controlled by a single recessive gene. With this background information, and with preliminary evidence that different strains of mosquitoes vary in their susceptibility to viruses (i.e., their ability to be infected and transmit the virus), another grantee has initiated studies to select strains of Aedes albopictus which are genetically resistant or susceptible to infection with dengue fever viruses. Demonstration of the genetic basis of resistance by fundamental genetic experiments could lead to an effective method of controlling this important human disease by replacing a susceptible vector mosquito in an area with a refractory strain of the same species or by increasing the resistance of a natural vector population by introducing into the population the gene controlling this factor.

Studies which began as basic taxonomy and were expanded to include fundamental biological processes have, during the past decade of support by the Institute, demonstrated the potential of aquatic parasitic fungi of the genus Coelomyces for control of mosquitoes, including those which transmit malaria and encephalitis. This investigator has now developed procedures for infecting large numbers of mosquito larvae to produce sufficient quantities of fungal sporangia for pilot field tests. Also, he has shown that a new species of the fungus will retain its viability when stored in the dry state for up to one year and that it is capable of infecting at least three different genera of mosquitoes. Additional basic studies (e.g., on the precise mechanism by which the fungus



TIB
IDB
L&K
COLLABORATIVE
RRB

infects the mosquito larvae and the possibility of development of resistance to the fungus) are proceeding as the investigator prepares for pilot field studies of one or more species of this fungus, which appears to be an obligate parasite only of the mosquito family and thus should be completely "safe" as a biological control agent.

RESEARCH GRANT HIGHLIGHTS

AI-09608-03 - A. R. Stevens

Dr. Stevens and a colleague have developed culture conditions that provide optimal, axenic growth of pathogenic strains of the protozoa Acanthamoeba and Naegleria. This will greatly facilitate the study of the factors responsible for virulence and which enable the pathogenic strains to invade mucous membranes.

AI-04717-12 - J. S. Remington

Dr. Remington's laboratory has continued to make major contributions to the understanding of the nature of Toxoplasma infection and the immune response to it. He has further demonstrated the value of the Toxoplasma model in the study of non-specific cellular resistance--an area of increasing interest in infectious diseases. In in vitro experiments, macrophages exposed to Toxoplasma antigen and lymphocytes from Toxoplasma-infected guinea pigs showed enhanced ability to kill Listeria organisms. The Toxoplasma model is particularly good for such studies, in view of the fact that non-specific cellular resistance appears to diminish with the disappearance of the antigenic stimulus from the host, since there is evidence for continued antigenic stimulation throughout the life of the Toxoplasma-infected host.

AI-07489-07 - J. K. Frenkel

Using the information obtained from his extensive studies on the biology, life cycle and epidemiology of Toxoplasma gondii, Dr. Frenkel has enunciated practical measures to be taken to prevent infection with this common pathogen. Heating of meat to 66°C kills the organism, and thorough washing of hands after handling of infected raw meat is an adequate safeguard. Various aspects of care of cats so as to avoid infection are of particular interest in view of recent widespread publicity about their role in transmission of the disease. If a cat's diet cannot be controlled, transmission of infection can be prevented by cleaning the cat's litter pan daily, flushing the feces down the toilet and disinfecting the pan with boiling water. Disposable gloves should be worn while performing these tasks. Common chemical disinfectants or drying are impractical for effective disinfection of cat feces.

AI-10627-03 - D. D. Despommier

Dr. Despommier's studies provide data to support the hypothesis that protective immunity to Trichinella spiralis is induced by exposure of the host to the secreted products of the developing larvae, in situ, and that these products emanate from alpha and beta secretory granules of the stichocyte. These alpha and beta granules are apparently synthetized between day 13 and 22 after

intramuscular injection of larvae, and they are secreted by the worms during the first 36 hours of life in the small intestine of the host, with approximately half of the granules released during the first 8 hours. There is no correlation between induction of protection and induction of eosinophilia. The latter is related to the presence of adult worms. A rapid (1 hr) and accurate serodiagnostic test has been developed; this employs counter-electrophoresis and an alpha and beta secretory granule-rich antigen fraction.

These studies, which have localized functional antibodies to a particular subcellular compartment of the parasite, are contributing much toward achieving objectives for projects in the Special Emphasis Area of Immunity to Animal Parasites: "...to isolate, identify and characterize the antigens responsible for..." humoral and cellular factors in the protective immune response in order to "...lead to more rational approaches to immunization, immunodiagnosis and the prevention of immunopathological events."

AI-09280-05 - P. E. Thompson

Dr. Thompson's studies continue to contribute to the elucidation of the mechanism of immunity to various filariae. He has accumulated further evidence that cell mediated immunity, and not humoral antibodies, plays the important role in dealing with filarial infections. He has developed procedures for harvesting substantial amounts of secretory-excretory antigens from Brughia pahangi $L_4$ stages maintained in cultures. Immunization experiments with this antigen are underway. Perhaps the most exciting finding of these studies is the demonstration that irradiated larvae of Dirofilaria or Brughia are unable to develop to maturity but induce a protective immunity. After preliminary studies had demonstrated that vaccination with 15 Kr-irradiated B. pahangi induced strong protection in dogs against subsequent homologous challenge but that the vaccine occasionally resulted in light infection, experiments were run to determine the proper level of irradiation to provide attenuated but viable larvae. The optimal dose was judged to be between 20 and 30 Kr. An experiment using 18 dogs is now underway using larvae of B. pahangi attenuated by 22.5 Kr.

AI-10129-03 - K. J. Bloch

During the course of studies on the immunobiology of the worm Nippostrongylus brasiliensis infection in the rat, Dr. Bloch examined mucosal extracts for antibodies to explore the role of local immune response in host resistance. While IgG1 antibodies were found in the mucosal extracts from day 20 onwards, IgA antibodies were not demonstrable at any time. Therefore, the influence of local gastrointestinal immunity was investigated with a simpler system in which rats were orally immunized with horseradish peroxidases and bovine serum albumin and absorption of these antigens was studied in vitro using the inverted gut sac technique. In comparison with controls, a consistent and significant decrease in peroxidase uptake was noted in both germ-free and conventional rats immunized with peroxidase; a similar decrease in albumin uptake was also noted in animals immunized with albumin. There was no difference in the uptake of an unrelated macromolecule. Local immunization seems to have interfered specifically with the intestinal uptake of

macromolecular antigens. These findings suggest a new role for the intestinal immune system: the limitation of uptake of intact protein antigens into the host. This could explain the high incidence of serum antibodies to ingested antigens in patients with impairment of gastrointestinal immune mechanisms, such as those with IgA deficiency.

AI-08163-07 - K. S. Warren

Dr. Warren and his colleagues have reported the production of a rabbit anti-mouse eosinophil serum (AES) with no cross-reactivity. The AES, obtained by immunizing rabbits with a highly purified suspension of eosinophils obtained from mice infected with Schistosoma mansoni, contained high titers of specific agglutinating and cytotoxic antibodies to eosinophils. Absorption with macrophages, lymphocytes and neutrophils did not affect the antieosinophilic titers; they were reduced markedly by absorption with eosinophils. One dose of AES (0.1 ml) injected into mice with schistosomiasis mansoni caused a mean decrease in circulating eosinophils of 90% within one hour. This was maintained for 5 days, after which there was gradual recovery. Availability of this serum should facilitate elucidation of the relationships among the different types of polymorphonuclear cells, the kinetics of eosinophils, the function of these cells, and their role in the pathogenesis of certain diseases.

## E. VIROLOGY AND RICKETTSIOLOGY

The approximate level of support given virology and rickettsiology:

| Type of Grant | Number | Amount |
|---|---|---|
| Research grants | 91 | $5,488,000 |
| Training grants | 25 | 1,612,000 |
| Fellowships | 22 | 415,000 |

### Summary

Many important questions on virus infections in man remain unanswered. A better understanding is needed of the role of viruses in human disease.

The two special emphasis programs of the Virology and Rickettsiology Branch, chronic and degenerative diseases and viral hepatitis, progressed this past year and further advances may be expected. In the area of viral hepatitis progress is being made in the isolation and culture of the hepatitis B (Serum Hepatitis) agent. This will result in improvement of blood screening in order to prevent infections by transfusion. It may also lead to a better understanding of clinical and epidemiological aspects of this disease. Support of influenza and other respiratory virus research continues. Vaccine development should provide improved protection in the near future.

### Research Grants Highlights

#### Influenza

Influenza, with its annual occurrence and predicted epidemics, is a problem which urgently demands solution. Development of safe and effective vaccine has been difficult because of the frequent and marked antigenic changes of the two types of influenza viruses involved. Procedures for the development of vaccines which will afford protection against epidemic-producing "wild" strains are evolving.

#### AI-09304-05 - E. D. Kilbourne

During the past decade the investigators at Mount Sinai have developed a genetic approach which should lead to improved understanding of the antigenic variation of influenza viruses. In collaboration with Baylor University scientists, they are continuing their studies on recombinant vaccines by immunizing volunteers against this disease. These studies have enabled them to predict that immunization will not prevent infection with the influenza virus but will block the evolution of infection to disease. Other studies leading to improved vaccines and their evaluation are being carried out.

#### AI-08831-05 - R. G. Webster

These studies provide evidence that new pandemic strains of influenza virus probably arise by recombination. Understanding the origin of new pandemic strains is paramount for a rational approach to the control or prevention of

the emergence of new human pandemic influenza viruses. Immediate practical applications from these studies are the production of "monospecific" antisera to the surface antigens of influenza for use in the accurate monitoring of antigenic variation in influenza viruses as these relate to production and evaluation of good vaccines.

AI-05600-11 - P. Choppin

Investigations supported on this grant have contributed significantly to our knowledge of virus structure, replication, and membrane biogenesis. Such knowledge will hopefully lead to eventual control of diseases produced by a number of the paramyxoviruses as well as influenza viruses. For example, the methods developed for isolation of the viral glycoproteins may be of direct application to safer and more acceptable vaccine production.

Common Cold

The debilitating infection known as the common cold (Common Viral Respiratory Infections) is associated with many viruses that have been isolated. Research data accumulated by persistent and excellent investigators is needed before practical control can be anticipated in any noteworthy degree. New techniques and approaches contribute to better understanding of underlying mechanisms of infectious agents and assure progress in prevention and cure.

AI-04059-11 - G. G. Jackson

Repetitive seasonal patterns of viral respiratory infections related to epidemiologic occurrence of these diseases have begun to emerge from studies at the University of Illinois Medical Center. Also some relationships among strain variations and clinical syndromes of disease have been recognized within some classes of viruses.

AI-09775-14 - J. P. Fox

Cross relationships among a number of the 37 rhinoviruses studied (90 rhinoviruses presently classified) have been found in investigations at the University of Washington. This encourages immunity studies although the significance of these cross-reactions is presently unknown.

Arboviruses

In spite of the developments following numerous years of research in this field that led to satisfactory control methods, the arboviruses continue to be an annual epidemic threat to large segments of our population. Continued research and surveillance are needed to keep abreast of the problems arising from these viruses as ecological conditions change and outbreaks ensue. This should lead to better control procedures where large human populations are exposed to this type of infection. It has been found that certain mosquitoes resistant to organophosphorous insecticides are no longer efficient encephalitis virus carriers. Detailed work with the arthropod-borne encephalitis viruses and their vectors continue to produce information favorable to prevention of these infections.

AI-02984-13 - P. Calindo

Rodents, and secondarily certain birds, appear to be the important reservoir hosts of enzootic Venezuelan Equine viruses in Panama. This makes continued surveillance and research essential to prevention of this potential threat to people in our southern states.

AI-03028-14 - W. Reeves

Studies on the resistance of Culex tarsalis (principal carrier of Western Equine Encephalitis) to licensed organophosphorous insecticides revealed some populations of this mosquito appear to be composed of virus-susceptible and resistant insects. The possibility of control of the competence of these mosquitoes to transmit virus could add much to the control of the arbovirus problems, and investigations are continuing on this.

AI-00771-17 - R. P. Hanson

Studies by investigators on this grant have shown that at least four antigenic variant viruses of California encephalitis virus group occur and are transmitted in Wisconsin. The LaCrosse virus, a member of the California group, is a significant cause of encephalitis in children. These investigators find evidence to suggest that the four closely related California group arboviruses may have evolved from a common ancestor in recent times and propose to test whether there is antigenic drift leading to these variants. If such variants have occurred recently, new ones might occur in the future and more virulent strains could be produced leading to an additional threat to people in the region. These continuing studies are necessary since the variant viruses apparently have different transmission times.

Herpes Viruses

Diseases caused by the herpes family of viruses are an increasingly serious problem in people whose normal immunologic responsiveness has been impaired either through naturally occurring life experiences or immunosuppressive treatment aimed at other problems. Research on these viruses is being supported to better understand the role of these organisms in relation to a number of infections in which they are suspect. Such research can lead to better control or refined therapeutic methods.

AI-06246-09 - J. G. Stevens

Herpes simplex virus has been shown to establish persistent infection in the trigeminal gangli of rabbits with recurrent eye infection. This supports the theory that in man these viruses are maintained in some quiescent state from which they may be reactivated to produce overt disease.

AI-03079-11 - T. Wright, Jr.

Since unusual dermatoglyphic findings have been observed in infants with congenital rubella infection, a study was designed to determine whether or

not unusual dermatoglyphics occur in patients with cytomegalic inclusion disease in infancy. It was found that infection with human cytomegalovirus has a teratogenic effect on the fetus during the first trimester of gestation as does rubella. Analysis of dermatoglyphics in 15 infants with cytomegalic inclusion disease revealed these unusual features in all infants. It is believed these indicate abnormal influences in gestation.

AI-09095-04 - J. Osborn

During investigations supported by this grant, significant thrombocytopenia during acute mouse cytomegalovirus infection was found directly associated with histologic, virologic, and immunofluorescent evidence of megakaryocytic infection and alteration by murine cytomegalovirus infection (MCMV). These studies on the effects of this virus infection on the hematopoietic elements of mice were instigated to ascertain the usefulness of this animal model in evaluating the effects of cytomegalovirus on hematopoietic (blood forming) systems in man. The importance of virus infection as an antecedent or direct cause of significant thrombocytopenia in children is widely assumed and it is important in congenital cytomegalovirus infection. No previous models of hematologic alterations induced by members of the herpes virus group have been previously reported, and these and future studies of the MCMV infection give encouragement for a better understanding of the mechanism of this component of cytomegalic inclusion disease.

Chronic and Degenerative Diseases

The accumulation of fundamental knowledge necessary for significant progress in understanding and overcoming the chronic and degenerative diseases of man comes only through difficult and time-consuming investigations on the viruses or virus-like agents known or suspected of causing these diseases. Research conducted on natural and experimental animal diseases is encouraging in that observations parallel conditions in chronic and degenerative disease of man and may give important leads for isolation, identification and route of transmission of infectious agents with encouragement for better management of these diseases.

AI-09118-05 - J. Craighead

Human diabetes mellitus is considered an inborn error of metabolism; however, the genetic defect ultimately responsible for its appearance remains unknown. The exact mode of inheritance is also open to question and geneticists have called the inheritance pattern a "nightmare." Since clinically evident diabetes in man has its onset more often in the adult than in the child, it is evident that environmental factors play an important role in the pathogenesis of the disease. For example, the genetic predisposition to diabetes may consist solely of an inability to replace worn out or diseased beta cells and reducing the production of needed insulin. With mice as animal models to study this disease it was found that when infected with the M variant of the encephalomyocarditis virus they develop necrotizing lesions of beta cells in the islets of Langerhans. Convalescent mice either recover, or exhibit abnormal glucose tolerance, or develop chronic hyperglycemia and a frank "diabetes-like" syndrome.

AI-06477-11 - J. Henson

Investigators on this project show progress in the elucidation of the pathogenesis of viral-induced arteritis in horses. They find the nature of these tissue reactions indicates that this may be comparable to tissue changes in man similar to those found in systemic lupus erythematosus and polyarteritis nodosa.

AI-09628-03 - F. Deinhardt

Independent confirmation of the isolation of an etiologic agent for human kuru in subprimate species (marmoset) has been accomplished on this project. This confirms similar cultivation in the chimpanzee by several other groups of investigators. It makes available an experimental animal model for study of such chronic diseases of the central nervous system which shows a much shorter incubation period of the agent and permits more rapid progress with investigations of this type.

Viral Hepatitis

The problems of viral hepatitis that are now designated as viral hepatitis A (epidemic or infectious hepatitis) and viral hepatitis B (serum hepatitis) continue as a disease of major public health importance with more than 60,000 reported cases and over 1,500 deaths each year in the United States. The disease is still the target of intensive research by a number of capable investigators with the greatest progress being made in the understanding of the infections involving the agent of viral hepatitis B. In this area advancements are being made in the understanding of the clinical, epidemiological and immunological behavior of the disease. Also progress appears in the isolation and culturing of this latter agent as well as rapid screening of blood to prevent infections by transfusion.

AI-09937-03 - D. Horstmann

Progress has been made in the purification and chemical analysis of the serum hepatitis antigen that will lead to better understanding of its infectious components, and help in future immunologic studies.

AI-10894-01 - I. L. Schweitzer

Findings on these investigations confirmed the presence of significant liver abnormalities in the children who are chronically hepatitis-B-antigen-positive with persistent transaminase elevations, delivered of mothers who had acute viral hepatitis during pregnancy or within six months of delivery. The fact that these patients were asymptomatic without physical signs of liver disease deserve emphasis. From these studies it seems that systematic hepatitis-B-antigen testing of all babies whose mothers have viral hepatitis during pregnancy or in the postpartum period are in order. Prolonged observations and repeated careful evaluations are continuing to ascertain the end-result of these hepatic lesions. The majority of newborns infected during the neonatal period remain hepatitis-B-antigen-positive and add to the overall numbers in the community.

AI-03874-13 - I. Gordon

Attempts at cultivation of hepatitis B virus in tissue culture continues to resist all-out efforts of a number of investigators. Investigations on this project have their greatest encouragement in their efforts to propagate human embryo liver cells. Preliminary results are encouraging as these are growing and may give a medium for tissue culture cultivation of this virus. Plans are to extend such culture trials to include primate inoculation and non-human primate tissue cultures.

Other Viruses

Investigations on a number of the other viruses are resulting in valuable information related to diagnostic laboratory support, clinical diagnoses, vaccine productions and reaction to vaccines, replication, isolation and characterization of the causal agents.

AI-01475-16 - E. H. Lennette

Although previous studies have shown that Colorado tick fever (CTF) virus occurs only transiently in the serum, but persists for prolonged periods in the blood cell fraction of man and experimental animals, the exact nature of this cell-associated viremia was unknown. These investigators have shown that persistent viremia is predominantly due to virus contained within erythrocytes. Erythrocyte precursor cells are presumably infected in the bone marrow and subsequently released into the blood stream, persisting for prolonged periods despite the presence of serum antibody. These findings point toward CTF virus being a useful model virus for further study of the mechanism of blood cell infection, with implications for other arboviruses or viruses believed to be involved in leukemia or other hematological disorders.

III. SPECIAL EMPHASIS PROGRAMS
DOLLAR ALLOCATION BY PROGRAM AREA

(Thousands; Direct Cost Only)

| Special Emphasis Programs | Fiscal Year 1973 | | |
|---|---|---|---|
| | Funds Allocated Amount | Approvals Funded from Allocation No. | Amount |
| Viral Hepatitis | $ 150 | 2 | $ 69 |
| Chronic and Degenerative Diseases of Man | 150 | 3 | 166 |
| Interferon | 112 | - | - |
| Mechanisms of Drug Resistance | 65 | 2 | 57 |
| Streptococcal Disease and Sequelae | - | - | - |
| Basic Biology of Venereal Disease | 200 | 9 | 247 |
| Biological Regulation of Vectors | 75 | - | - |
| Immunity to Animal Parasites | 75 | 4 | 143 |
| Lymphocyte Biology | 100 | - | - |
| Mechanisms of Immunity to Infectious Agents | 100 | 1 | 57 |
| Cell-Mediated versus Humoral Antibody Responses | 200 | - | - |
| Clinical Immunology 1/ | - | - | - |
| Total, Special Emphasis | $1,227 | 21 | $739 |

1/ Approved without fund allocation; November 1972 Council



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IV. RESEARCH GRANTS PROGRAM ANALYSIS

| Fields | 1973 Estimate Amount |
|---|---|
| Allergy and Immunology | $16,500,000 |
| Asthma & Allergic Disease Center | (1,400,000) |
| Lymphocyte Biology | (1,500,000) |
| Clinical Immunology and Immunopathology | (2,000,000) |
| Mechanisms of Immunity to Infectious Disease | (840,000) |
| Selective Activation of Cell-Mediated Versus Humoral Antibody Responses | (890,000) |
| Bacterial and Mycotic Diseases | 17,948,000 |
| Streptococcal Disease and Sequelae | (1,550,000) |
| Mechanisms of Drug Resistance | (770,000) |
| Basic Biology of Venereal Disease | (900,000) |
| Viral Diseases and Problem Areas | 13,500,000 |
| Interferon and Other Antiviral Substances | (1,100,000) |
| Chronic and Degenerative Diseases of Man | (627,000) |
| Viral Hepatitis | (973,000) |
| Parasitic Diseases and Problem Areas | 6,700,000 |
| Immunity to Parasites | (1,500,000) |
| Biological Regulation of Vectors | (975,000) |
| Subtotal | $54,648,000 |
| International Centers for Medical Research | 2,399,000 |
| Total, Research Grants | $57,047,000 |

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COLLABORATIVE RESEARCH PROGRAMS


Summary 1

Transplantation and Immunology Branch

1. Summary 5
2. Program Status Report 6
3. Contract Narratives 11
4. Publications 37

Research Resources Branch

1. Summary 42
2. Program Status Report 44
3. Contract Narratives 51
4. Publications 77

Infectious Disease Branch

1. Summary 78
2. Program Status Report 80
3. Contract Narratives 90
4. Publications 149

COLLABORATIVE RESEARCH PROGRAMS: SUMMARY

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

FY 1973

Fiscal 1973 was a year which witnessed significant changes within NIAID Collaborative Research Programs including new appointments and a major reorganization. Dr. George J. Galasso was named Chief, Infectious Disease Branch, and Dr. John E. Nutter became Chief, Research Resources Branch. Following return from his special training period at Johns Hopkins University School of Public Health, Dr. Daniel I. Mullally was appointed Special Assistant to the Scientific Director for Coordinating review of human clinical trials conducted through the contract mechanism.

As of May 1, 1973, an NIAID reorganization separated Collaborative Research from Direct Operations (Office of the Scientific Director) and Dr. Howard A. Minners, who had served as Chief, Geographic Medicine Branch since it was first organized within NIAID in 1968, was appointed Associate Director for Collaborative Research. Collaborative Research now consists of four Branches, namely, Geographic Medicine Branch, Infectious Disease Branch, Research Resources Branch and the Transplantation and Immunology Branch. Presently included in the Office of the Associate Director for Collaborative Research are: Dr. Robert J. Byrne, Assistant Associate Director for Collaborative Research, Dr. Daniel I. Mullally, Special Assistant for Contracts Involving Human Subjects and Mrs. Doris Parkinson, Administrative Officer for Collaborative Research. Dr. Earl S. Beck was appointed Chief, Geographic Medicine Branch replacing Dr. Minners.

Due to an increased workload and limited personnel resources, training activities were somewhat restricted in FY 1973. Training included special courses in computer technology, radiologic health, technical writing and administration. Staff also participated actively in the Upward Mobility College.

Collaborative Programs continued to utilize their established advisory committees for both evaluation of ongoing scientific efforts and for program redirection. There was increasing reliance on individual committee members and non-government scientists in the review of contract proposals. New committee management regulations implementing P.L. 92-463 require that, to the maximum extent possible, meetings be open to the public, and that there be advance public notification of committee meetings. While new requirements have not affected the conduct of committee meetings, the newer regulations did however influence us to abolish subcommittees including the Subcommittee on Influenza.

Progress in the Infectious Disease Branch during FY 1973 includes the implementation of clinical trials to evaluate exogenous interferon, iododeoxyuridine (IUD) and adenine arabinoside (Ara-A) in the treatment of disseminated herpes zoster and herpes simplex and herpesvirus encephalitis;

initiation of trials to determine the value of hyperimmune HB Ab gamma globulin in preventing hepatitis B development and increased utilization of the hepatitis testing laboratory; initiation of hepatitis epidemiologic studies and beginning of a larger scale, phased testing of polysaccharide vaccines, including vaccines against pneumococcal pneumonia. Collaborative studies to find a safer and yet effective means of immunizing against smallpox in the United States have been completed, and the data are being currently analyzed. Significant scientific progress was made in hepatitis research as a result of close collaboration involving NIAID, and intramural scientists from Bureau of Biologics (FDA), the National Institute of Neurological Diseases and Stroke and those from the Molecular Anatomy Laboratory, and the Scripps Institute. As a result of this collaboration, it was possible to achieve serial passage of hepatitis B virus in rhesus monkeys, to titrate hepatitis B virus in chimpanzees and to study the pathogenesis of hepatitis. Collaborative efforts also generated new knowledge on the nature and distribution in tissues of hepatitis B antigens and their precursers and on the nature of the "Dane" particle which may be the etiologic agent of hepatitis B.

Other significant scientific findings generated through contracts, agreements and NIAID intramural research are now being applied. For example, temperature sensitive (ts) strains of influenza virus developed in the Laboratory of Infectious Diseases, NIAID, are being tested as an attenuated virus vaccine for influenza. Information developed from contract-supported studies on polysaccharide vaccines against bacterial meningitis indicates that infants respond to the vaccines, and it now appears possible to immunize those children in the high risk age group.

During FY 1973 the Transplantation Immunology Program initiated a study designed to assess the value of histocompatability matching in determining success in transplanting kidneys from unrelated cadaver donors and recipients. The study, involving six transplant centers and a centralized data collecting center is expected to produce a thorough analysis of approximately 800 transplants/year in which donors and recipients tissues were typed using standard NIH materials. The study has been made possible by prior years efforts to acquire and analyze HLA typing sera and select the most potent and well-defined sera for filling the standard tissue typing trays. Also the direct result of past Transplantation Immunology Program efforts has been the initiation of a controlled clinical trial of antilymphocyte globulin (ALG) as a means of preventing or mitigating rejection of transplanted organs. These studies have been made possible by past investment in developmental efforts to solve three interrelated problems: 1) the best source of immunizing antigen (thymocytes), 2) the animal of choice for raising antisera (rabbits) and, 3) the most appropriate animal model for testing potency (rhesus monkeys and chimpanzees).

The sponsorships of workshops and publication of catalogs describing typing sera and related reagents furnishes information which is essential to clinical application in transplantation surgery as well as furthering research in transplantation immunology.

The Research Resources Program has essentially completed the production, testing and packaging of reagents to the well-established viruses and mycoplasmas of human health significance and is now progressing to programs on allergens, immunologic reagents and antisera against highly purified viral antigens.

The hepatitis reagents produced by the Research Resources Branch have been increasingly valuable for research on HB Ag subtypes and have had direct application in epidemiologic studies where knowledge of subtypes was instrumental in defining the spread of infection. High-titered antisera to a number of isolated neuraminades and hemagglutinins of influenza A viruses have already been produced and the complete spectrum of antisera to the 26 known hemagglutinins and neuraminades will be complete within two years. These reagents will be valuable in epidemiological research and in studies on antigenic shifts in human influenza virus.

## Publications

Byrne, R.J. Neurotropic Viral Diseases (of the horse). Chapter in Equine Medicine and Surgery, American Veterinary Publications, Inc. Santa Barbara, California, 1972.

Byrne, R.J. The Control of Eastern and Western Arboviral Encephalomyelitis of Horses. 3d International Conference on Equine Infectious Diseases, Paris, France. JAVMA (in press).

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# TRANSPLANTATION AND IMMUNOLOGY PROGRAM

## 1. SUMMARY

Understanding the phenomenon of transplant rejection is of immense importance to many facets of clinical medicine, and to the field of cellular immunology in general. Through its Reagents and Techniques Program, the Transplantation and Immunology Branch (TIB) is investigating the complex procedures of histocompatibility matching in order better to understand and control the immunological phenomena which are involved in organ transplantation. Program contractors have demonstrated the importance of platelet complement fixation and mixed lymphocyte culture techniques. The validity of histocompatibility typing has been improved through the utilization of reliable antisera and through the use of multiple procedures by several investigators. The reliability of reagents being distributed by the program is insured by testing in expertly qualified laboratories, and by a continuing review of the results of tissue typing in more than one hundred laboratories using a uniform technique with identical reagents on a standardized "NIH" tray. Considerable effort has been devoted to the improvement of techniques for the freeze-drying of reagents; the present systematic program for the lyophilization of frozen serum will result in a majority of the serum becoming available in a stable form which can be shipped at ambient temperatures.

The Immunosuppression Program of the TIB sponsors contracts to evaluate drugs and antilymphocyte serum which show promise as immunosuppressants. Subhuman primate skin graft studies indicate that rabbit antihuman thymocyte globulin (ALG) is a very effective agent. Large quantities of this material have been produced for use in a clinical trial of ALG.

Quantities of purified HL-A and H-2 soluble antigens have been produced; these are used by a number of investigators in animal studies to induce tolerance in experimental organ transplant models. These materials have been used to immunize laboratory animals to produce high-titer antisera needed for experimental histocompatibility typing.

One contract studies the immunopathology of kidneys in transplant patients. Results indicate that there is an increase in rheumatoid-like factor in transplant recipients and that antigen-antibody complexes may be deposited in the kidney and other organs in these patients.

One of the most important missions of the TIB is to collect and distribute information on reagents, typing techniques, organ transplants and on the efficacy of tissue matching. The program supports registries, sponsors workshops and scientific meetings and, through the publication of contractor's reports and the issuance of catalogs, typing manuals and periodic statistical reports, provides a valuable resource of scientific and technical information for scientists and for the public.

## 2. PROGRAM STATUS REPORT

During fiscal year 1973 approximately 1.3 million dollars were spent on contracts and agreements in support of Transplantation and Immunology Programs. These efforts can be considered to fall into six general program categories.

### I. Reagents and Techniques Program

Since its inception in 1965, much progress has been made by the Collaborative Transplantation and Immunology Program in developing the methodology for histocompatibility typing and in making accessible the quality reagents necessary for this work. The current emphasis of the nine contracts in this field are to compare various techniques of tissue typing in their ability to define immunologic differences between individuals, to produce a continuing supply of high-quality histocompatibility typing sera; to make available these sera in bulk and in microquantities on a standardized tray; to develop new typing techniques that are potentially simpler or more accurate than those currently employed; to conduct research directed at discovering new antigen specificities; and to provide other reagents used in histocompatibility research. Nine contracts which have been supported for work in one or more of the above areas remained in effect through all or part of FY'73. Their annual level of funding was $520,023.

Refinements of typing techniques have been studied on four contracts. A comparison of cross-matching techniques is underway to determine the most sensitive approach to this crucial matching procedure. A controlled study of the efficacy of lymphocytotoxicity and fluorochromasia techniques is in progress. The platelet complement fixation test has been shown to be a simple, easily readable typing technique, which can be automated. The importance of mixed lymphocyte reactions in the matching of donors and recipients is being evaluated, as are several other procedures designed to detect immunologic discrepancies between individuals. Neutrophil specific antigens have been discovered; antisera have been produced, and made available through the NIAID serum bank for study in bone marrow transplant donor-recipient pairs. Improved techniques and reagents have resulted in the discovery of new antigens; several of the typing contracts have made contributions in this area.

The NIAID typing serum bank currently contains over 1,200 sera with a total volume of 234 liters. Additions to the bank in FY'73 included 83 sera obtained by contracts from 5 laboratories, 62 donated by users of the bank and 27 purchased outright by the Branch. Quality control analyses of the sera prior to their procurement were performed by 7 typing laboratories; this system has increased the overall quality of the additions to the bank. Usage of the bank remained heavy with 997 shipments dispensed to 289 investigators during 1972.

Through the use of a standardized tray and uniform lymphocytotoxicity typing techniques, a continuing effort to maintain the quality of reagents and the reproducibility of laboratory results is underway. Over

15,000 trays were delivered to over 112 U.S. laboratories for clinical organ typing in calendar year 1972. During 1973 significant improvements in the clinical typing tray have been made, with expansion to 72 wells, defining 30 specificities by means of 68 well-studied, reliable antisera.

Preliminary steps were taken during FY-73 to produce and distribute trays filled with lymphocytes from well-characterized individuals. These will provide a standard profile of histocompatibility antigens for use in quality control of sera, and for the assessment of patient immune sensitivity.

Reagents other than human typing sera have been produced by TIB contract for use in histocompatibility research. Through these contracts human complement components and inhibitors have been available to qualified investigators, as have soluble mouse histocompatibility antigens (described in Section V).

## II. Evaluation of Tissue Matching in Renal and Bone Marrow Transplantation

Although the utility of tissue typing has been demonstrated in matching genetically related kidney donors and recipients, a problem still remains in evaluating the efficacy of these procedures in the selection of appropriate cadaver kidney donors. Well-matched donor-recipient pairs seem to have fewer episodes of threatened graft rejection than recipients receiving kidneys from completely mismatched donors, but convincing evidence is lacking; this trend is even less clear in the case of bone marrow transplants. Nine TIB contracts totalling $558,852 were in effect during FY'73 in this program area.

The analyses of the data based on a simple count of mismatched antigens have yielded confusing results due in part to the difficulties in collecting accurate clinical and typing data. The consensus to date is that a correlation will be found between closeness of matching and renal function in unrelated donor-recipient transplants. The Typing Evaluation Study of the TIB is designed to answer that crucial question by means of a controlled collaborative study. This will not only advance the understanding of clinical transplant immunology, but will also provide necessary scientific justification for the expensive typing procedures now in use throughout the country. This project is now possible in part because of the NIH typing tray which has afforded the means for standardized tissue matching. Through the seven participating contracts a large population of accurately matched cadaver renal transplants (over 800 annually) will be studied with detailed clinical followup. A search for 4-antigen perfect matches will be emphasized.

This FY'73 marks the final year of NIAID support for the American College of Surgeons/National Institutes of Health Transplant Registry contract. The Branch Chief has been the project officer for the ACS/NIH Kidney Registry and head of the NIH Transplantation Committee which provides general supervision for this contract. The Registry contract may continue to be funded through other Institutes of the NIH.

In addition to its contract-supported efforts, the TIB conducts its own program for the collection of fundamental information on transplants. On an annual basis, serum users are asked to provide data indicating the numbers of transplants of different organs conducted by the various hospitals for whom they provide typing services. The data are collated and used by the Branch in assessing priorities for the distribution of serum, and is distributed to other serum users, to the ACS/NIH Registry, and to the coordinator of the collaborative Typing Evaluation Study. Experience in the past has shown that this mechanism has been far more successful in obtaining transplantation information than other registries. During 1972, the Branch collected information indicating that more than 4,800 kidney transplants had been performed in 405 hospitals around the world.

III. Immunosuppression Program

The Branch has sponsored five contracts totalling $637,016 per year for the study and evaluation of potentially active immunosuppressive drugs and sera in animals and patients. These contracts help to develop techniques for the prevention or the abrogation of graft rejection. Cyclophosphamide and a variety of new anti-neoplastic drugs are currently being investigated. Studies to date in animals and patients indicate that cyclophosphamide is an effective agent for the prevention of graft rejection in bone marrow transplantation. A method for the treatment of bone marrow graft recipients with cyclophosphamide has been developed and is in general usage in the U.S. and Europe. Other anti-tumor agents (methotrexate, cytosine arabinoside and L-asparaginase) have been used both individually and with antilymphocyte serum and have been shown to be effective in preventing first and second set graft rejection. Important methodology has been developed to evaluate immunosuppressives.

Antilymphocyte serum (ALS) and purified antilymphocyte globulin (ALG) are of the current interest as means of preventing and/or mitigating rejection. Over 35 percent of the U.S. transplantation programs utilize these preparations. Unfortunately, few controlled studies have been conducted to evaluate the effectiveness of these products. The TIB has established through contract the only facility in the western hemisphere for the mass *in vivo* testing of ALS and ALG. This contract has screened more than 150 preparations, many of which are currently being used in a clinical setting. The two most effective antigens for the production of ALS are thoracic duct and thymus cells; ALS raised in rabbits appears to be superior to that produced in horses in its ability to prolong skin grafts in Rhesus monkeys and chimpanzees. Preclinical pharmacology and toxicity studies are nearly completed on a product which will be used in a controlled, double-blind, 100-patient clinical trial at the University of California. The high cost of subhuman primate testing has prompted the search for effective *in vitro* techniques of assaying ALS and ALG. Studies indicate that rabbit antihuman thymocyte serum is effective only if it exhibits a high *in vitro* lymphocytotoxic titer. This technique is being utilized to exclude non-effective rabbit antisera from the pool of material for clinical trial.

## IV. Immunopathology Program

The Immunopathology Program supports with $99,500 per year the study of immunological changes that occur in tissues and serum of experimental animals and human patients who have received organ transplants and/or immunosuppressive therapy. Laboratory animals have been used to develop models of kidney diseases, techniques to recognize antigen-antibody reactions involved in kidney diseases, and to study the graft rejection process. Immunohistological changes in transplanted and rejected renal grafts are being investigated to assess whether graft failure is due to recurrent nephritis, to the deposition of circulating immune complexes in the renal tubules or glomeruli, or to an immunologic histoincompatibility reaction. Studies involving antilymphocyte globulin (ALS) preparations used in immunosuppressive therapy have revealed that many of these agents derived from solid lymphoid organ lymphocytes contain anti-glomerular basement membrane (GBM) antibodies.

## V. Antigen Program

The Branch has sponsored two contracts through their final year (at $140,000) for the study of soluble murine histocompatibility antigens. These have established the methodology for the production of quantities of soluble antigens for laboratory investigation. Several methods of antigen solubilization and fractionation are being employed to produce active stable fractions which are then assayed by a variety of in vitro and in vivo techniques. These antigen fractions are also provided to NIH intramural, contract and grantee investigators to aid them in the study of such promising immunological phenomena as immune enhancement and tolerance.

## VI. Organ Preservation Program

Until FY'73 the TIB was supporting a multifaceted attack on the problems of long-term organ preservation, an effort which lead to the development of more effective cryoprotective media, improved techniques for thawing frozen solid organs, and progress in the use of hyperbaria during the freezing process. When it became clear that considerable fundamental basic research is in order in this field before applied research can be useful, this contract effort was phased out. Support at $83,847 yearly remains for work in cell freezing and serum freeze-drying procedures which have immediate practical benefit to nation-wide tissue matching efforts.

## VII. Publications and Conference Support

As part of its information program, the Branch prepares an annual updating of the Catalog of Tissue Typing Antisera. The last issue of this Catalog was distributed in January 1973 to approximately 700 users. This Catalog, which is over 900 pages in length, contains information on the 1,200 sera which are available from the Institute's bank. It lists the availability of serum and a great deal of information concerning the specificity,

utility, titers and the frequency of reaction with the various racial groups. It is the only such document available for use by transplantation immunologists. The Manual of Tissue Typing Techniques was revised during this fiscal year. This contains up to the minute information on the various tests which may be used for histocompatibility matching, and is distributed to investigators and used in the tissue typing courses. The Branch also provides catalogs of other reagents, including H-2 (mouse) antisera, complement components, and soluble antigen fractions.

Contract funds have provided partial support for the planning and conduct of the 4th International Congress of the Transplantation Society held at San Francisco in September 1972. Also with contract support the WHO Expert Committee on Histocompatibility conducted studies on antigens in racial isolates to search for and determine the frequency of exotic lymphocyte antigens. As the result of these studies, discussed at a workshop in Evian, France, valuable new information was developed pertaining to newly discovered antigens and "splits" of currently recognized antigens. The report of this workshop is pending. This information will lead to a better understanding of the HL-A system and of tissue typing in general.

TRANSPLANTATION AND IMMUNOLOGY BRANCH

3. CONTRACT NARRATIVES

ALBERT EINSTEIN COLLEGE OF MEDICINE, YESHIVA UNIVERSITY (PH43-68-1295)

Title: Preparation of soluble purified H-2 isoantigens

Contractor's Project Director: Dr. Stanley G. Nathenson

Project Officer: Dr. John G. Ray, Jr.

Objectives: This contract provided soluble mouse H-2 antigens to selected qualified investigators who were interested in immunogenicity and immunological tolerance. Funds were also provided for storage and distribution of these antigens and to improve the efficiency of the solubilization and purification processes.

Major Findings: During the past five years this investigator has processed approximately 240,000 mouse spleens from seven different strains of mice to produce soluble H-2 histocompatibility antigens in various stages of purity. Thus far, sixty-three investigators have utilized approximately 2,800 milligrams of these soluble antigenic fractions in support of their research activities. These soluble H-2 alloantigens have been shown to be immunogenic and to modify graft-versus-host reactions.

Significance to NIAID Program and Bio-Medical Research: Basic knowledge concerning the immunogenicity and tolerogenicity of mouse H-2 soluble histocompatibility antigens and their relationship to accelerating and inhibiting graft rejection have been demonstrated. This is in agreement with similar studies of human HL-A soluble antigens. The ultimate goal is to develop methods to abrogate the graft rejection process and permit permanent graft survival.

Proposed Course of Project: This contract was terminated June 23, 1973, the current inventory of soluble H-2 antigen is sufficient to allow new investigators to acquire these as standard preparations. The methodology has been published and many scientists now prepare their own soluble H-2 antigens.

Date Contract Initiated: June 24, 1968

Current Annual Level: Prior year support

ALBERT EINSTEIN COLLEGE OF MEDICINE (NIH69-2071)

Title: Human neutrophil-specific antibodies

Contractor's Project Director: Dr. Parviz Lalezari

Project Officer: Dr. Robert C. Scott

Objectives: This contractor is providing the Institute Serum Bank with human neutrophil-specific antisera and is searching for new such antisera through a program of screening potential donors. Once obtained, he is measuring the activity of these antisera against neutrophils and lymphocytes. These antisera are used clinically by the contractor and by a growing number of other investigators particularly in conjunction with bone marrow transplantation.

Major Findings: Neutrophil-specific antigens have been shown to be rare but are of clinical significance in neonatal neutropenia and have been implicated as a factor in the rejection of bone marrow grafts. Women with infants having neonatal neutropenia have been shown to be the only practical sources of these antisera in high titer, and among this population the contractor has uncovered the four distinct neutrophil specificities which are known at present, and has contributed each in quantity to the NIAIDSerum Bank. Two of the antigens are apparently alleles. Solubilization of one of the antigens has been accomplished and the chemical identification of each antigen is under investigation.

Significance to NIAID Program and Bio-Medical Research: The search for neutrophil-specific antigens is a field of research in which this contractor has been the sole participant. Evidence is now accumulating from many sources that these antigens may well be of great importance in determining the success or failure of bone marrow transplants, which are being performed in increasing number for severe leukopenia of various etiologies. The activity of these antigens against renal or other solid organ transplants is also under study. Definition of the genetics of neutrophil antigens and the further determination of their pathological significance is of pressing clinical importance to transplantation and undoubtedly to medical immunology in general.

Proposed Course of Project: Terminates August 31, 1973

Date Contract Initiated: June 27, 1969

Current Annual Level: Prior year support ($44,000)

AMERICAN FOUNDATION FOR BIOLOGICAL RESEARCH, BIOMEDICAL RESEARCH INSTITUTE
(NIH-NIAID-73-2508)

Title: Freeze-drying of typing sera and processing, freezing and quality control of lymphocyte cells

Contractor's Project Director: Mr. Vernon P. Perry

Project Officer: Mr. Donald B. Hare

Objectives: This contractor freeze-dries the human histocompatibility typing sera for the NIAID Serum Bank, serves as a reference laboratory on certain of the antisera to study the effects of freeze-drying and continues the work on freeze-drying of lambda quantities of typing sera in the Falcon Tray. In addition, the contractor processes and freezes aliquots of lymphocyte cells from bulk quantities of human blood, makes cells available for redistribution into typing trays, and monitors the quality of frozen cells and trays.

Major Findings: Sixty-eight liters of the Bank's inventory has been freeze-dried in one ml quantities during this fiscal year. Their cell panel has been increased to thirty-four. The contractor has done the majority of the titering of the antisera before and after freeze-drying to determine whether or not freeze-drying caused a loss of activity or specificity of a serum. Freeze-drying of one lambda quantities of antisera in the Falcon Tray was temporarily discontinued. Lymphocytes have been obtained from whole blood, frozen, stored in liquid nitrogen and checked for viability. The quality of NIH frozen cell trays has been determined.

Significance to NIAID Program and Bio-Medical Research: The freeze-dried typing sera will eliminate much of the expense and many of the logistical difficulties in the shipping of sera and NIH Typing Trays. The freeze-dried antisera can be stored at -20°C, and much of the -70°C freezer space can be eliminated with all of its inherent problems. Freeze-drying of an antiserum seems to prolong its shelf life, and is thereby of value to the Bank.

The NIH frozen cell trays enable transplant centers to type a recipient's blood for preformed antibodies with a superb cell panel containing all the major and minor histocompatibility antigens. The tray will also be a good tool in finding better and scarce antibodies in a screening program, and also it can be used for quality controlling of promising antisera.

Proposed Course of Project: Continuation through September 30, 1973

Date Contract Initiated: March 1, 1968

Current Annual Level: $119,408

BIONETICS RESEARCH LABORATORIES, INC. (PH43-68-1327)

Title: A primate pre-clinical test center for immune study, primarily with ALS

Contractor's Project Director: Dr. Clement C. Darrow

Project Officer: Dr. Donald E. Kayhoe

Objectives: The principal objectives in this contract are to maintain a sub-human primate facility for the testing of human antilymphocyte serum (ALS) and to operate an immunology laboratory to conduct a battery of *in vitro* tests. This year, principal emphasis was placed on *in vivo* testing of program-produced rabbit anti-human thymus serum, which was readied for clinical trials.

Major Findings: During the past year 18 antilymphocyte preparations were tested. A battery of *in vitro* techniques were used and all preparations were administered to Rhesus monkeys in *in vivo* tests to measure their ability to prolong the survival of skin grafts. Thirty-four monkeys were used in this testing. The most effective material tested was a rabbit antihuman-thymus serum produced by the U.S. Navy Contract, which is being used in the current clinical trial. ALS with steriods and imuran producedlonger graft survivals than ALS alone in some studies. Ten-day courses of therapy were shown to produce longer graft survivals than the same material given over a shorter period of time.

Significance to NIAID Program and Bio-Medical Research: ALS was used for immunosuppression in more than half of the kidney transplants performed in the U.S. during 1972. The only satisfactory method, in our opinion, to test the efficacy of ALS is by measuring its ability to prolong homograft survival in sub-human primates. This contract provides facilities for such testing. Studies of the efficacy and toxicity of material produced for a large scale, double blinded, clinical trial have been tested during the past year, and such a human study is now underway.

Proposed Course of Project: This contract was terminated on June 27, 1973. Any additional testing required will be done under an exchange of funds agreement with the Naval Medical Research Institute, Bethesda, Maryland.

Date Contract Initiated: June 28, 1968

Current Annual Level: $212,000

UNIVERSITY OF CALIFORNIA (NIH-NIAID-72-2540)(NO1 AI 22540)

Title: Evaluation of ALG as an immunosuppressive agent

Contractor's Project Director: Dr. Folkert O. Belzer

Project Officer: Dr. Robert C. Scott

Objectives: This contract provides for the collection and recording of clinical and laboratory information on 100 kidney transplant patients participating in a double blind clinical trial of a standardized, pretested rabbit antihuman thymocyte globulin. The study, done in collaboration with the National Naval Medical Center, is designed to test the suitability of such ALG more rigorously, objectively, and convincingly than has been done in the past.

Major Findings: Patients will be entered on the study over the first two years of the contract, and the course of each will be followed under this contract until two years after the last of the 100 patients have been transplanted. At present the study has just begun; consequently, there is as yet insufficient data for preliminary analysis.

Significance to NIAID Program and Bio-Medical Research: More than one-third of the clinical transplant centers in the U.S. reportedly utilized some form of antilymphocyte serum as an immunosuppressive. Objective evidence as to the value of this practice is scarce and controversial. Several preparations in use have failed to demonstrate significant skin graft prolongation in subhuman primates. The present study is designed to provide some of the first reliable, objective data from which the actual effects of such a drug can be judged. This contract gathers the data for the study.

Proposed Course of Project: Continuation through March 1977; presently funded through February 28, 1974.

Date Contract Initiated: June 29, 1972

Current Annual Level: Prior year support ($120,647)

CLEVELAND CLINIC FOUNDATION (PH43-68-1298)

Title: Human tissue typing and transplantation laboratory

Contractor's Project Director: Dr. William E. Braun

Project Officer: Dr. Robert C. Scott

Objectives: This contractor maintains a histocompatibility typing facility for the Cleveland area which serves as a quality control laboratory for NIAID typing reagents. In addition, he has provided the NIAID Serum Bank with high quality HL-A antisera through a screening program. The contractor is also involved in the comparison of two variations of lymphocytotoxicity typing: one utilizing peripheral blood lymphocytes, the other using lymph node as the source of cells to be typed. Among HL-A identical pairs, related and unrelated, he is investigating the value of the mixed leucocyte reaction to insure immunologic identity.

Major Findings: This contractor has developed an efficient typing program and has collected a panel of high quality antisera to be used in conjunction with the NIH tray in clinical typing. The quality of his work is high and for this reason he has become the primary quality control laboratory for the identification of new sera for the NIAID Serum Bank. He also evaluates random samples of freshly produced NIH trays to insure their quality. The contractor's own antiserum screening program, involving primarily multiparous women, has itself provided the Serum Bank with several valuable antisera. His clinical experience accumulated thus far largely from related donors and recipients supports data from other laboratories showing better graft survival with matched than with mismatched renal transplants. In addition, he has found it practical to uncover apparently hidden HL-A antigens by trypsinzation of the cells and is continuing his investigations into this potentially valuable technique. His recent findings have indicated that the typing of cadaver lymph node cells yields fewer complete typings than does typing of peripheral blood cells from living subjects. Possible explanations for this clinically important observation are being pursued.

Significance to NIAID Program and Bio-Medical Research: This contract has contributed greatly to the efforts of the Branch to assess the efficacy of tissue typing in selected organ donors and recipients. It has been particularly productive in the procurement of new sera for the Serum Bank. The Contractor's highly reliable performance in typing is basic to his quality control function,an essential part of the Branch's effort to constantly improve the serum reagent stock of the Bank. He has been a leader in efforts to perfect the practical techniques of matching donors and recipients for kidney transplantation.

Proposed Course of Project: Continuation through December 27, 1973

Date Contract Initiated: June 28, 1968

Current Annual Level: $97,300

CLEVELAND CLINIC FOUNDATION (NIH-NIAID-72-2536)

Title: Evaluation of histocompatibility matching in renal transplantation

Contractor's Project Director: William E. Braun, M.D.

Project Officers: Dr. Daniel Mullally and Mr. Donald B. Hare

Objectives: To gather data in collaboration with other regional transplant centers to be forwarded to the University of North Carolina, School of Public Health for analysis to determine the value of histocompatibility matching in renal transplantation. To collect and store cells and sera from donors and recipients.

Major Findings: This contractor has collected information on approximately 40 patients. This data will be made available to the University of North Carolina statistical contractor.

Significance to NIAID Program and to Bio-Medical Research: A major emphasis of the Transplantation and Immunology Branch has been the development of histocompatibility typing techniques. Although typing is a widely used and accepted procedure, it has not been shown that the degree of tissue matching correlates with the success of a cadaver renal transplant. This study is designed to answer this important and very basic question.

Proposed Course of Project: This contract terminates December 28, 1973.

Date Contract Initiated: June 29, 1972

Current Annual Level: $14,756

COMMUNITY BLOOD COUNCIL OF GREATER NEW YORK (PH43-67-1334)

Title: Histocompatibility testing of donors and recipients of organ grafts

Contractor's Project Director: Dr. Fred H. Allen, Jr.

Project Officer: Dr. Robert C. Scott

Objectives: This contractor was engaged in the evaluation of tissue typing as a means of selecting the donors and recipients of organ transplants within the New York City area.

Major Findings: This contractor utilized a two-stage lymphocytotoxicity test and a panel of 56 sera to conduct tissue typing on a 24 hour basis in the New York City region. Using this method they accomplished as many as 900 typings, representing approximately 50 renal transplants, annually. Data on a limited number of transplants suggested a longer survival with better-matched kidney grafts. Their data have helped to rule out any possibility of close genetic linkage between most of the common RBC antigens and the HL-A antigen system.

Significance to NIAID Program and Biomedical Research: This contract afforded an opportunity not only to determine the importance of histocompatibility "leucocyte" typing in the selection of donors and recipients of organ grafts, but also correlated RBC and serum protein groups with the results of typing and with the clinical course. Their study of the efficacy of typing in selecting unrelated graft donors is encouraging when viewed by the haplotype analysis, but their moderate case-load proved to be insufficient to yield a statistically significant conclusion on this point.

Proposed Course of Project: Terminated October 31, 1972

Date Contract Initiated: June 29, 1967

Current Annual Level: Prior year support ($53,400)

4th INTERNATIONAL CONFERENCE OF THE TRANSPLANTATION SOCIETY (NIH-72-G-455)

Title: Arrange and administer Fourth International Congress of the Transplantation Society

Contractor's Project Director: Dr. Samuel Kountz

Project Officers: Dr. Donald E. Kayhoe and Mr. Willard Hill

Objectives: This contract provided partial support for the Fourth International Conference of the Transplantation Society in San Francisco September 25-29, 1972. The conference served as a forum for all responsible investigators to report their new developments in transplantation.

Major Findings: Three major scientific activities were conducted during the Fourth International Congress. Plenary sessions, in which major areas of basic and clinical research in transplantation immunology were discussed by recognized leaders and responsible investigators in the field; scientific sessions, in which brief communications of new developments were presented, and workshops where in depth discussions of research were held on cellular immunology, the graft rejection process and tumor immunology. The results of this meeting were published in Transplantation Proceedings, Volume V (1), March 1973.

Significance to NIAID Program and Bio-Medical Research: The Transplantation and Immunology Branch provides funds for support of scientific meetings in areas of program interest to aid in the rapid dissemination of research information. This was the official forum for the Transplantation Society, an international organization whose members comprise the principle group active in transplantation. The Transplantation and Immunology Branch has provided partial funding for previous Congresses held in 1970, 1968 and 1966.

Proposed Course of Project: Terminated February 2, 1973

Date Contract Initiated: February 3, 1972

Annual Level of Funding: $34,831

HARVARD UNIVERSITY (NIH-69-90)

Title: H-2 histocompatibility antigen extraction, purification and characterization.

Contractor's Project Director: Dr. Paul S. Russell

Project Officer: Dr. John G. Ray, Jr.

Objectives: This contractor produced water soluble H-2 antigens from mouse tissue and characterized the reactions of these antigens by determining their *in vitro* and *in vivo* biological activity. Quantities of solubilized antigen were produced and distributed to qualified investigators as directed by the project officer. This contractor also conducted comparative *in vivo* biological tests with soluble H-2 antigens produced by another contract.

Major Findings: This contractor has produced sufficient quantities of soluble H-2 antigens to meet program needs. Thus far, twenty-seven qualified investigators have requested and received these antigens. They have been shown to be immunogenic and have enhanced skin graft survival in congenic strains of mice. This contractor has been successful in separating the d and k region antigens from solubilized mouse tissue. *In vivo* biological tests have shown that the k region antigens possessed greater immunogenic properties than the d region antigens.

Significance to NIAID Program and Bio-Medical Research: The mouse tissue histocompatibility antigens (H-2) are needed in the Transplantation and Immunology Branch program to learn more about their relationship to the human (HL-A) histocompatibility antigens and the methods for inducing tolerance in mice and men. The procedures for the extraction and purification of soluble HL-A and H-2 antigens are similar but the *in vivo* testing for graft prolongation can better be accomplished in the genetically well-defined mouse model.

Proposed Course of Project: This contract will terminate on September 8, 1973. A sufficient supply of contractor produced soluble antigens is available to permit continuation of studies by independent investigators.

Date Contract Initiated: April 9, 1969

Prior Year Support: $112,669

THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS (NIH-NIAID-72-2529)

Title: Evaluation of histocompatibility matching in renal transplantation

Contractor's Project Director: Dr. Olga Jonasson

Project Officers: Dr. Daniel Mullally and Mr. Donald B. Hare

Objectives: To gather data in collaboration with other regional transplant centers to be forwarded to the University of North Carolina School of Public Health for analysis to determine the value of histocompatibility matching in renal transplantation. To collect and store cells and sera from donors and recipients.

Major Findings: This contractor has collected information on approximately 95 patients. This data will be made available to the University of North Carolina statistical contractor.

Significance to NIAID Program and to Bio-Medical Research: A major emphasis of the Transplantation and Immunology Branch has been the development of histocompatibility typing techniques. Although typing is a widely used and accepted procedure, it has not been shown that the degree of tissue matching correlates with the success of a cadaver renal transplant. This study is designed to answer this important and very basic question.

Proposed Course of Project: This contract terminates December 28, 1973.

Date Contract Initiated: June 29, 1972

Current Annual Level: $38,890

IRWIN MEMORIAL BLOOD BANK OF THE SAN FRANCISCO MEDICAL SOCIETY (PH43-68-945)

Title: Operation of a regional leukocyte phenotyping facility in a community blood bank

Contractor's Project Director: Dr. Herbert A. Perkins

Project Officer: Dr. Robert C. Scott

Objectives: This contractor is comparing the techniques of typing by lympnocytotoxicity and fluorochromasia in the ability to detect histocompatibility antigens on peripheral blood lymphocytes. He also conducts a screening program to provide histocompatibility typing antisera for the NIAID Bank, and he has served as a quality control laboratory to evaluate candidate sera for the Bank. He has conducted histocompatibility typing for one of the largest renal transplant centers in the U.S., and has studied the relevance of such tissue matching to graft survival.

Major Findings: Preliminary studies on the relative merits of lymphocytotoxicity vs. fluorochromasia have suggested that fluorochromasia may be the more sensitive technique by a slight margin. A more rigorously controlled study is underway to clarify this point. The contractor has demonstrated that kidney cell typing will occasionally uncover HL-A factors and incompatibile cross-matches which were missed with the use of lymphocytes. He has shown that in renal transplantation involving siblings graft survival has correlated well with haplotype matching; in the case of cadaver-donor transplants the same trend exists but tne data are not conclusive.

Significance to NIAID Program and Bio-Medical Research: Although the active nature of tne renal transplant program served by this contractor facilitates his efforts to compare the clinical result with that predicted by histocompatibility data, the issue of typing efficacy is as yet unanswered. Because this is a question of great importance, the TIB has launched a new program in this area. Fiscal 1973 has been the last year of funding for this contract; it terminates with the completion of its techniques-comparison study. Other functions are to be absorbed in ongoing contracts of the Branch.

Proposed Course of Project: Terminates December 27, 1973

Date Contract Initiated: March 27, 1968

Current Annual Level: $79,861

THE JACKSON LABORATORY (PH43-66-438)

Title: The production, storage and distribution of mouse alloantisera

Contractor's Project Director(s): Drs. George Snell and Marianna Cherry

Project Officer: Dr. John G. Ray, Jr.

Objectives: This contractor is to produce, store and distribute mouse H-2 histocompatibility and certain non-histocompatibility antisera to qualified investigators. He is to periodically check these sera for potency of reactivity and specificity. Information sheets on each sera are to be updated in lieu of new information concerning their usefulness. In addition, this contractor is to provide approximately 80,000 spleens annually from among the following inbred strains of mice: $C_{57}$BL/6, BALB/c, $C_{57}$Bl/10Su, $B_{10}D_2$-new/Su, $C_3$H/He, and A/J. These mouse spleens are to be shipped to another contractor who extracts the soluble H-2 antigens.

Major Findings: This contractor has supplied 35 different scientific investigators with approximately 800 ml of mouse H-2 alloantisera for use in studying immunogenetics of the histocompatibility loci and in graft rejection during fiscal year 1973. Approximately 20,000 spleens were delivered to Dr. Stanley Nathenson for starting material in solubilizing the mouse H-2 antigen during FY'73.

Because of the interest in basic cellular immunology and in graft survival in the mouse, the past year has been devoted to acquiring additional sera for distribution. These antisera depict antibodies to the -d and -k end of the H-2 gene through the use of different congenic lines of mice. Twelve of these sera are currently being distributed to qualified researchers.

Significance to NIAID Program and Bio-Medical Research: These reagents are used to characterize soluble H-2 fractions of antigens, type mouse tissue cell lines, and for studying tumor and cellular immunology. These antigens will enhance our knowledge of immunogenicity and tolerogenicity and have a direct application to the HL-A antigenic system in man. Efforts are being made to control the tissue rejection process in this mouse model so that a similar control can be acquired in human organ transplantation.

Proposed Course of Project: Continuation through April 30, 1974

Date Contract Initiated: June 1, 1966

Current Annual Level: $235,000 (FY'71 funds - three years)

JOHNS HOPKINS UNIVERSITY (PH43-66-924)

Title: Factors related to survival of bone marrow allografts in man

Contractor's Project Director: Dr. George W. Santos

Project Officer: Dr. Donald E. Kayhoe

Objectives: This contract evaluated and correlated methods of histocompatibility testing and immunosuppression with the clinical results of allogenic bone marrow grafts in man and rodents and studied the efficacy of methotrexate and cyclophosphamide as drugs for the prevention and treatment of bone marrow graft rejection.

Major Findings: The graft vs. host phenomenon presents a major impediment to successful bone marrow transplantation. In the animal model, the contractor has developed a system for the administration of cyclosphosphamide as a means of preventing or partially reversing rejection. Using this technique, he has conducted a series of human bone-marrow transplants. Problems continue to exist in the management of these patients; graft versus host disease, while of less severity, still occurs; systemic infections present the most serious problems and are often fatal. Cyclophosphamide treatment, developed by this contractor, has been used with reported success for kidney transplants by a number of surgeons. New regimens of treatment are being studied in rodents in an effort to find more selectively immunosuppressive means of patient management and hopefully to eliminate the problems of intercurrent infections.

Significance to NIAID Program and Bio-Medical Research: Patients with far-advanced hematological malignancies and those with other bone marrow deficiency states constitute a potential group for transplantation. Survivals to date, especially in leukemics, have been rare. The development of methods for suppressing rejection which permit the maintenance of the patients' ability to respond to infections should result in markedly increased survival rates. This contract also afforded a unique opportunity to study immunologic activity of the transplanted marrow cells in the graft vs. host reaction and to assay the relative importance of the histocompatibility antigens. Because of the excessive mortality rate in leukemic patients who were given marrow transplants, the investigators have greately reduced the number of human studies and devoted their principal efforts to the study of the animal models.

Proposed Course of Project: This contract was terminated on June 30, 1973, after a six-month extension of funds to permit an orderly phase-out. The contractor was encouraged to seek graft support for the continuation of his animal studies.

Date Contract Initiated: June 29, 1966

Current Annual Level: $40,014

UNIVERSITY OF LEIDEN (PH43-65-992)

Title: Search for HL-A sera and improvement of histocompatibility techniques

Contractor's Project Director: Dr. Jon J. van Rood

Project Officer: Dr. Donald E. Kayhoe

Objectives: This contract seeks to further evaluate the efficacy of the platelet complement fixation, agglutination, and cytotoxic tests for studying histocompatibility antibodies. These techniques are used in selecting donors and recipients in the Eurotransplant Program. This includes transplant units in Holland, Belgium and West Germany that attempt to find the most compatible donor-recipient pairs for transplantation. Organ recipients are then followed to determine the prognostic value of histocompatibility matching. Further, the contract provides funds to search for and collect quantities of antisera containing histocompatibility antibodies and to perform testing procedures on sera submitted by the NIAID to confirm the presence of various histocompatibility antigens. This contract also provides funds to develop and test more efficient automated techniques for reading and recording the results of complement fixation and cytotoxicity tests for tissue typing.

Major Findings: The contractor has developed a panel of cell donors which are used in a program to find monospecific platelet complement fixation test serum and to discover new specificities. He has deposited 12,090 cc of unique histocompatibility typing serum in the bank during the past fiscal year. Comparisons are being carried out in the Eurotransplant Program to demonstrate the relative efficacy of the complement fixation and the microcytotoxicity techniques for histocompatibility typing. Eurotransplant Program recipients of matched cadaver kidneys have had fewer rejection episodes and present less of a management problem than those who receive kidneys with two or more antigens mismatched. Because of the homogenicity of the Netherlands population, the likelihood of achieving a perfect match is greater than in the heterogeneous U.S. population. This makes it possible to achieve more well-matched transplants in Holland.

Significance to NIAID Program and Bio-Medical Research: This contractor has an excellent facility for obtaining good serum and is the principal source for reagents for the platelet complement fixation tests. This procedure makes it possible to greatly increase the number of typings done by a single technician. The platelets used in the test are readily transportable and survive for long periods of time. The complement fixation test lends itself to automation. The contractors use a photomultiplier tubes to read the results of individual tests afford a rapid and reproducible system for typing. The contractors continued progress will ultimately result in the development of a complete set of reagents for this procedure.

Proposed Course of Project: Continuation through December 31, 1973

Date Contract Initiated: June 24, 1965

Current Annual Level: $59,370

MEDICAL COLLEGE OF VIRGINIA - VIRGINIA COMMONWEALTH UNIVERSITY (PH43-68-659)

Title: Analysis of antigenic and serologic factors important in human organ transplantation

Contractor's Project Director: Dr. James C. Pierce

Project Officer: Dr. Robert C. Scott

Objectives: This contractor has been engaged in the study of renal post-transplant patients to determine whether the presence of histocompatibility antibodies may be used as a means of predicting graft rejection. He has also performed a comparative study of four cross-matching techniques, i.e. the standard lymphocytotoxicity, the trypsinized cytotoxicity, the kidney cell immune adherence, and the kidney mixed antiglobulin tests.

Major Findings: The contractor has demonstrated that kidney cell immune adherence cross-matching will occasionally demonstrate presensitization to a prospective donor in the face of a negative lymphocytotoxicity cross-match. According to present knowledge of the genetics of the HL-A system his findings can account for the observed incidence of hyperacute and acute rejection among recipients of renal transplants. Refinements of these techniques and a potentially more sensitive technique were added to the evaluation during this final year of the contract.

Significance to NIAID Program and Bio-Medical Research: In the present state of knowledge the one immunologic test that is considered absolutely crucial in preparation for a kidney transplant is the cross-match to determine whether the recipient has pre-existing antibodies to the antigens of the proposed graft. Perfecting the technique for this determination is therefore of great importance, and has been the major mission of this contract. The meaningful completion of the comparison study during this last contract year is a significant practical contribution to the art of clinical renal transplantation.

Proposed Course of Project: Terminates December 31, 1973

Date Contract Initiated: January 15, 1968

Current Annual Level: $52,302

MICROBIOLOGICAL ASSOCIATES, INC. (NIH-71-2443)

Title: Preparation and evaluation of human histocompatibility typing trays

Contractor's Project Director: Robert-John Trapani, Ph.D.

Project Officer: Dr. John G. Ray, Jr.

Objectives: This contract provides facilities for the production of preloaded histocompatibility typing trays which contain pre-typed cells or sera. The contractor also performs quality control testing on tray or bulk sera.

Major Findings: This contractor has produced 14,250 preloaded sera histocompatibility typing trays, evaluated 114 unknown sera for antibodies, and quality controlled 70 standard typing trays during this fiscal year. He increased his standard panel from 20 to 60 well-characterized cells. A 72-well sera histocompatibility typing tray was prepared and delivered to 12 investigators for analysis. Preliminary reports indicate that these trays are acceptable for further distribution.

Significance to NIAID Program and Bio-Medical Research: The employment of preloaded standard typing trays containing the best obtainable sera and cells makes it possible for U.S. transplanters to use identical reagents for typing of donors and recipients and to test for HL-A antibodies in the recipients serum. This reagent standardization facilitates the use of rare high-quality histocompatibility typing antisera and provides statistically comparable results which will aid in the exchange of organ program as well as the analysis of the importance of histocompatibility matching in organ graft survival. The contract provides a quality control facility necessary for continued high quality standardization of tissue typing sera. The innovative 72-well tray enables the tissue typing laboratory to type for 30 defined HL-A antigens rather than the 24 antigens available in the earlier 60-well tray. The standard trays containing well-defined HL-A cells (lymphocytes) enable the immunologist to seek preformed antibodies in the graft recipient's serum and in HL-A quality control.

Proposed Course of Project: To continue to June 1974

Date Contract Initiated: June 1971

Current Annual Level: $77,786

UNIVERSITY OF MINNESOTA (NIH-NIAID-72-2530)

Title: Evaluation of histocompatibility matching in renal transplantation

Contractor's Project Director: Edmond J. Yunis, M.D.

Project Officers: Dr. Daniel Mullally and Mr. Donald B. Hare

Objectives: To gather data in collaboration with other regional transplant centers to be forwarded to the University of North Carolina School of Public Health for analysis to determine the value of histocompatibility matching in renal transplantation. To collect and store cells and sera from donors and recipients.

Major Findings: This contractor has collected information on approximately 158 patients. This data will be made available to the University of North Carolina statistical contractor.

Significance to NIAID Program and to Bio-Medical Research: A major emphasis of the Transplantation and Immunology Branch has been the development of histocompatibility typing techniques. Although typing is a widely used and accepted procedure, it has not been shown that the degree of tissue matching correlates with the success of a cadaver renal transplant. This study is designed to answer this important and very basic question.

Proposed Course of Project: This contract terminates December 28, 1973.

Date Contract Initiated: June 29, 1972

Current Annual Level: $70,090

UNIVERSITY OF TEXAS, M.D. ANDERSON HOSPITAL AND TUMOR INSTITUTE (PH43-68-949)

Title: Suppression of immune responses with drugs and biological products; A search for more effective less myelotoxic immunosuppression

Contractor's Project Director: Dr. Evan M. Hersh

Project Officer: Dr. Donald E. Kayhoe

Objectives: This contract provides funds to evaluate new drugs and antilymphocyte serum for their immunosuppressive activity in transplant recipients and cancer patients; the contractor also develops new methodology for the evaluation of immunosuppression, and studies the immune state in patients with uremia and a variety of malignant diseases.

Major Findings: The contractor has investigated a number of drugs and antilymphocyte serum which are currently being used in kidney and heart transplant patients. He has developed standardized methods for the measurement of immune responsiveness and has used these techniques to study patients prior to, during and after therapy. A number of new anti-tumor agents are being evaluated.

Significance to NIAID Program and Bio-Medical Research: The information gained concerning the utility of new immunosuppressants has been most helpful in the management of the post-transplant patient. The standardization of the methodology makes it possible for investigators to compare the utility of drugs in producing immune paralysis. The techniques will be most valuable when attempts are made to induce immune tolerance, since the aim of this procedure is to produce specific tolerance to histocompatibility antigens sparing immunologic reactivity to other antigens.

Proposed Course of Project: Funded through January 12, 1974 (terminal year)

Date Contract Initiated: June 21, 1968

Current Annual Level: $122,600

NAVAL MEDICAL RESEARCH INSTITUTE, NATIONAL NAVAL MEDICAL CENTER (Interagency Agreement)

Title: Preparation and testing of anti-human thymocyte serum

Contractor's Project Director: Dr. Kenneth W. Sell

Project Officer: Dr. Donald E. Kayhoe

Objectives: This contract provides partial support for a program to evaluate the efficacy of rabbit anti-human thymocyte globulin (RATG). A double blind comparison of the RATG with a normal human albumen control is being conducted at the University of California, San Francisco in 100 kidney transplant patients. This contract provides funds for the collection and evaluation of the study of the data. The National Naval Medical Center has obtained a Division of Biologics, FDA Investigational New Drug License and will be responsible for the conduct of the clinical trial.

Major Findings: Optimum methods for the production and testing of rabbit anti-human thymus globulin have been developed. A clinical supply of RATG has been produced and packaged and the clinical study is underway.

Significance to NIAID Program and Bio-Medical Research: This facility has produced RATG of demonstrated efficacy in the prolongation of skin and kidney transplants in subhuman primates. Material has been produced under strict quality control procedures to assure potency and purity for the clinical trial. Studies in the past with material used by other investigators has revealed that a high percentage of ALS preparations do not significantly prolong the survival of subhuman skin grafts. Active antilymphocyte serum is needed as an additional measure to prevent human homograft rejection. The RATG produced under this contract permits a study to determine the efficacy of potent ALS.

Proposed Course of Project: The clinical trial will require approximately four years. This will permit two years to enter patients on the study and a minimum of two years to follow-up.

Current Annual Level: $150,000

UNIVERSITY OF NORTH CAROLINA SCHOOL OF PUBLIC HEALTH (NOI AI 32515)

Title: Evaluation of efficacy of tissue matching in renal transplants

Contractor's Project Director: R. W. Helms, Ph.D.

Project Officers: Dr. Daniel Mullally and Mr. Donald B. Hare

Objectives: Collection and analysis of data on human kidney transplants from a large group of collaborating institutions in six regions. This study is designed to determine the efficacy of histocompatibility matching in renal transplants.

Major Findings: The contract was not awarded until March 7, 1973 due to budgeting and personnel restrictions. The contractor has developed a reporting form, which has undergone preliminary field testing in the six regions. It has also been sent to OMB for tentative approval. Five sub-committees have been appointed for the study and are in operation. They are: 1) Study Design and Data Analysis, 2) Criteria and Definitions, 3) Lab Methods and Standards, 4) Forms, and 5) Publications and Publicity.

Significance to NIAID Program and to Bio-Medical Research: A major emphasis of the Transplantation and Immunology Branch program has been the development of histocompatibility typing techniques for use in renal transplantation. Although these procedures are now widely used and accepted, the question has not been answered as to whether or not the degree of matching correlates with the success of a cadaver renal transplant. The answer to this important and very basic question is the main objective of this program; however, many other important factors will be studied in this program, such as, 1) effects of associated diseases on the recipient, 2) blocking antibodies, and 3) recurrence of original diseases.

Proposed Course of Project: This contract terminates March 6, 1974.

Date Contract Initiated: March 7, 1973

Current Annual Level: $176,972

UNIVERSITY OF ROCHESTER (NIH-NIAID-72-2539)

Title: Evaluation of histocompatibility matching in renal transplantation

Contractor's Project Director: Dr. Allyn G. May

Project Officers: Dr. Daniel Mullally and Mr. Donald B. Hare

Objectives: To gather data in collaboration with other regional transplant centers to be forwarded to the University of North Carolina School of Public Health for analysis to determine the value of histocompatibility matching in renal transplantation. To collect and store cells and sera from donors and recipients.

Major Findings: This contractor has collected information on approximately 58 patients. This data will be made available to the University of North Carolina statistical contractor.

Significance to NIAID and Bio-Medical Research: A major emphasis of the Transplantation and Immunology Branch has been the development of histocompatibility typing techniques. Although typing is a widely used and accepted procedure, it has not been shown that the degree of tissue matching correlates with the success of a cadaver renal transplant. This study is designed to answer this important and very basic question.

Proposed Course of Project: This contract terminates December 28, 1973.

Date Contract Initiated: June 29, 1972

Current Annual Level: $40,150

SCRIPPS CLINIC AND RESEARCH FOUNDATION (PH43-68-621)

Title: Study of the etiologic events and antigen-antibody systems in human glomerulonephritis and the course of nephritis in transplanted patients

Contractor's Project Director: Dr. Frank J. Dixon

Project Officer: Dr. John G. Ray, Jr.

Objectives: This contractor studies serum and renal tissue samples from nephritic patients prior to, during and after kidney transplantation. He studies the occurrence of circulating antiglomerular basement membrane (AGBM) antibodies and their presence in antilymphocyte globulin (ALG). He investigates the role of microbial agents in chronic glomerulonephritis, of antigen-antibody complexes in immune complex nephritis, and the recurrence of the original nephritis in the transplanted kidney.

Major Findings: The contractor studied 933 serum and renal tissue specimens from 551 patients in 51 centers during the past fiscal year. In 66% of the specimens, either glomerular immunoglobulin indicative of immune complex glomerulonephritis, deposits of complement, or linear glomerular deposits indicative of AGBM antibodies were reported to the patient's physician. Over 50% of the transplanted patients having AGBM-antibody-induced nephritis developed recurrent glomerulonephritis; in some this was severe enough to cause graft failure. Patients receiving HL-A identical transplanted kidneys developed glomerulonephritis only when their original renal disease was glomerulonephritis. Less well matched patients occasionally evidenced immune complex glomerulonephritis without prior glomerulonephritis. Two patients produced antibodies against tubular basement membrane antigens.

Significance to NIAID Program and Bio-Medical Research: The methodology and collaboration developed in this contract have contributed to a better understanding of the rejection process and the immunopathogenesis of glomerulonephritis. All of this information has proven very useful in the management of the transplant patient and in determining the cause of their renal failure.

Proposed Course of Project: Continuation through March 1, 1974

Date Contract Initiated: January 2, 1968

Current Annual Level: $99,500

UNIVERSITY OF TEXAS, SOUTHWESTERN MEDICAL SCHOOL AT DALLAS (NIH-NIAID-72-2531)

Title: Evaluation of histocompatibility matching in renal transplantation

Contractor's Project Director: Dr. Peter Stastny

Project Officers: Dr. Daniel Mullally and Mr. Donald B. Hare

Objectives: To gather data in collaboration with other regional transplant centers to be forwarded to the University of North Carolina School of Public Health for analysis to determine the value of histocompatibility matching in renal transplantation. To collect and store cells and sera from donors and recipients.

Major Findings: This contractor has collected information on approximately 148 patients. This data will be made available to the University of North Carolina statistical contractor.

Significance to NIAID Program and to Bio-Medical Research: A major emphasis of the Transplantation and Immunology Branch has been the development of histocompatibility typing techniques. Although typing is a widely used and accepted procedure, it has not been shown that the degree of tissue matching correlates with the success of a cadaver renal transplant. This study is designed to answer this important and very basic question.

Proposed Course of Project: This contract terminates December 28, 1973.

Date Contract Initiated: June 29, 1972

Current Annual Level: $69,310

UNIVERSITY OF UTAH (NIH-NIAID-72-2537)

Title: Evaluation of histocompatibility matching in renal transplantation

Contractor's Project Director: Dr. C. W. DeWitt

Project Officers: Dr. Daniel Mullally and Mr. Donald B. Hare

Objectives: To gather data in collaboration with other regional transplant centers to be forwarded to the University of North Carolina School of Public Health for analysis to determine the value of histocompatibility matching in renal transplantation. To collect and store cells and sera from donors and recipients.

Major Findings: This contractor has collected information on approximately 20 patients. This data will be made available to the University of North Carolina statistical contractor.

Significance to NIAID Program and Bio-Medical Research: A major emphasis of the Transplantation and Immunology Branch has been the development of histocompatibility typing techniques. Although typing is a widely used and accepted procedure, it has not been shown that the degree of tissue matching correlates with the success of a cadaver renal transplant. This study is designed to answer this important and very basic question.

Proposed Course of Project: This contract terminates December 28, 1973.

Date Contract Initiated: June 29, 1972

Current Annual Level: $21,070

UNIVERSITY OF WASHINGTON (PH43-67-1435)

Title: Evaluation of tissue typing techniques in bone marrow transplantation

Contractor's Project Director: Dr. E. Donnall Thomas

Project Officer: Dr. Robert C. Scott

Objectives: This contractor studies the laboratory techniques of immunologic matching as applied to the donors and recipients of bone marrow transplants. He evaluates these techniques in relation to the clinical course of the transplant patient. Techniques presently under study include HL-A serotyping, family typing (genetic studies), mixed leukocyte culture, cell-mediated lympholysis, neutrophil agglutination, and mixed antiglobulin reactions. In addition he supplies the NIAID Serum Bank with typing antiserum through an active screening program involving multiparous women.

Major Findings: During this last reported year, the contractor has studied 652 subjects including 143potential marrow recipients and their families, far more than any other investigator in the field. Forty-four patients received marrow grafts following extensive matching procedures. Serotyping and MLC have been shown to be necessary procedures in this setting, but they are not sufficient in themselves, for HL-A identical, MLC-negative sibling donor grafts have demonstrated graft-vs-host (GVH) disease. Knowledge is actively being pursued as to the relevance of other tests in predicting immunologic compatibility.

Significance to NIAID Program and Bio-Medical Research: Bone marrow transplantation has been shown to be a promising treatment for a number of hematologic, neoplastic, and immunologic deficiency diseases. The key to clinical success lies in understanding and controlling the immunologic factors involved in rejection of the graft, or rejection of the host by the graft (GVH). This contract continues to provide new information which is essential for the optimal matching of marrow transplant patients. Hopefully such efforts will increase the availability of this form of treatment to include the majority of needy patients rather than merely the fortunate few.

In addition, the serum procurement feature of the contract helps the NIAID Serum Bank to maintain the continued essential function of the typing reagent program.

Proposed Course of Project: Continuation through December 28, 1973

Date Contract Initiated: June 29, 1967

Current Annual Level: $114,582

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## RESEARCH RESOURCES BRANCH

### 1. Summary

The need for well characterized reference reagents as an adjunct to research is currently well accepted. At its inception Research Resources Branch began by providing seed virus preparations and corresponding antisera as a way of facilitating research in virology. Since that time the work of the program has expanded until now the Branch provides a selected variety of well defined reagents to the research community in those specific instances where they are vitally needed but are not available from other sources. These reagents for health research purposes include viral and mycoplasma seed cultures and their corresponding antisera, allergens and interferons. All reagents are characterized by appropriate microbiological, immunological and biochemical methods and provide recipients with reference materials for the conduct of research. The program also provides resources in support of research requiring biophysical instrumentation, particularly zonal centrifuge systems. These facilities have proven to be especially valuable to the intramural staff and has resulted in highly productive cooperative efforts.

As a result of past efforts, reagent sets for most of the accepted enteroviruses are available, but during this year, the Lim Benyesh-Melnick typing pools A-H were certified for distribution and the formulation of pools J-P was determined. Production and certification of reagents for the rhinoviruses continues and at this point the acquisition of the 89 serotypes comprising phases I and II is at an advanced stage; production of only six sets is still in progress and certification activities are proceeding on schedule. During this year some adenovirus seeds and antisera were remade and reagent sets for the recognized human types (1-31) are now available. A highlight of this year was the acquisition for distribution of the appropriate antiserum and reference antigen for subtyping hepatitis B antigen Y (ay+ d-). These reagents in combination with previously available ones for D (ad+ y-) subtype specificity are useful as a set for subtyping HBAg, and in addition, production for other HBAg serotypes is in progress. The arbovirology program continues to provide reagents to high priority virus types and during this fiscal year the program certified 21 additional products making a total of 137 arbovirology reagents available for distribution.

Although the mycoplasma production and certification program is complete, these reagents are currently being assessed by a network of WHO/FAO Laboratories, and these data will increase greatly the value of these materials. The myxovirus program this year had two major accomplishments; a synthetic substrate for the assay of neuraminidase was obtained, and a production contract was negotiated which will result in the production of monospecific antisera to all the known hemagglutinins and neuraminidases from influenza viruses of man and animals.

The allergen program has been given considerable emphasis during this fiscal year. A contract was initiated for the production of short ragweed antigens E, K and Ra 3 and work on these antigens is progressing on schedule. Arrangements have been made to purchase 10,000 grams of perennial ryegrass pollen and RFP's distributed for extracting group antigens I, II and III. RFP's have also been issued for the production of Asc-1 antigen from *Ascaris suum* and the production

of a polyvalent rabbit antiserum against short ragweed pollen.

The research community is made aware of the availability of reagents through the distribution of the Catalog of Research Reagents, supplemental lists of reagents, announcements in professional publications and the participation of staff members in program related national and international scientific meetings. Advice in identifying the need for and feasibility of producing reagents is obtained through established committees, special consultant groups, communications with intramural and extramural scientists, and the conduct of Branch-sponsored workshops. A planning committee is presently formulating the program for a herpesvirus workshop to be conducted in the fall of 1973 to determine the need for additional reagents.

## 2. PROGRAM STATUS REPORT

### Enteroviruses

Most of the known enteroviruses are now available for distribution and as a result of past activities the monkey antisera are now recognized by WHO as an international reference reagent. During this fiscal year seed preparation for coxsackie A-17 and seed and antisera for enterovirus type 68 were produced at the Center for Disease Control (CDC) through an interagency agreement. These products are presently being assayed by the enterovirus certification laboratory. This certification laboratory continues to be located at the California State Department of Public Health and is also involved with identification of prospective new types, assays related to determination of useful shelf-life of stored reagents and assays for the formulation of Lim Benyesh-Melnick (LBM) typing pools. Plans are now complete to have reagents for a new enterovirus type (Toluca 1) produced during the next fiscal year through an interagency agreement with CDC.

The LBM typing pools (A-H) were certified for distribution and have now received wide distribution and appear to be a useful adjunct to enterovirus research. Plans have now been completed and formulas for 7 additional typing pools (J-P) against group A coxsackieviruses have been finalized. These various pools are scheduled for processing and packaging during the last part of FY 73. Subsequent to certification these will then be added to the reagents available for distribution.

### Arboviruses

This program continues its production activities for those arboviruses of demonstrated and potential importance to disease on the North American continent. Two contractors are presently engaged in the production of reference reagents (seed virus, immune ascitic fluid and control ascitic fluid) to ten arboviruses. Production is at various stages of completion for the following reagent sets: St. Louis Encephalitis, Russian Spring Summer Encephalitis, Japanese B Encephalitis, Powassan, Semliki Forest, Piry, Mucambo, VEE (Fla.), Dengue 2 and Dengue 3 viruses. It is anticipated that the majority of these reagent sets will be produced within the next fiscal year and that certification will be performed on most of the bulk reagent material. This program is also presently supporting research and development work on the production of Dengue reagents in rats and it is anticipated that this contract will develop methodology for producing high quality immune ascitic fluids in more useful volumes for these viruses.

The certification laboratory for this program continues to be located at the Yale Arbovirus Research Unit and this facility provides the Branch with the full array of assays needed to fully characterize the seed viruses and ascitic fluids of this program. The certification laboratory has now tested an overall total of 137 arbovirus reagents which were subsequently recommended for distribution by the Arboviruses Subcommittee. In addition, as part of its FY 73 activities the certification laboratory has also tested 27 bulk reagents which were approved for packaging and an additional 23 bulk reagents are in the process of being tested prior to final processing.

The program continues to provide support to the Catalogue of Arthropod-borne Viruses of the World through an interagency agreement with the Center for Disease Control. This activity provides a very useful mechanism for interchange of information among international research laboratories involved in work with this large and diverse group of infectious agents.

A contract was awarded during FY 73 for remaking three mosquito blood meal antisera. These antisera are being prepared against the blood of ciconiiform (heron), columbiform (pigeon), and passeriform (sparrow/starling) birds. When these are complete there will be a total of 12 antisera available for distribution. There are antisera against four major animal groups in the array and this provides investigators with a technique for identifying the source of blood in field-collected mosquitoes.

Included in this program is a Mammal Identification service for processing collections and specimens submitted from NIH affiliated laboratories and institutions. Research with arboviruses characteristically involve wild rodents and other mammals and the necessity for providing a firm taxonomic basis for field and laboratory studies is imperative. This facility is located in the National Museum of Natural History, Smithsonian Institution. During the first 9 months of FY 73 this service has provided 1,045 identifications and a key to the non-volant mammals of lower Amazonia. The key is prepared using no technical language so that professionally untrained field workers can identify mammals from this area of importance to arbovirology research.

Mycoplasma

Seed and antiserum to twenty-eight mycoplasma species of human and non-human origin are currently available for distribution. During FY 73 an extensive WHO/FAO testing program was initiated on these NIH reference reagents. It is anticipated that the information obtained from the thirty laboratories participating in this study will substantially increase the value of these reagents by further definition of reactivity, titer, etc., under an expanded variety of test conditions.

Branch staff conferred in January 1973 with Drs. Shmuel Razin, Israel; David Taylor-Robinson, England; A. E. Freundt, Denmark; and several intramural staff members concerning potential reagent needs for mycoplasma research. This group determined that two potential areas of need are reagents for human T mycoplasmas and antisera for three exotic mycoplasmas (Mycoplasma mycoides, capri and agalactiae). These suggestions will be discussed with the Research Resources Branch Advisory Committee at its next meeting and appropriate action taken.

Rhinoviruses

The Phase I rhinovirus production program (types 1-55) has been completed with the exception of type 55. This single type has presented a continuing problem to the producing laboratory but at this time production of the seed virus is now in the final stage. Three of the five phase II (types 56-89) contractors have completed their requirements and have shipped their bulk materials which are now stored for subsequent processing and packaging. One

of the phase II contractors whose work is now terminated produced seed reagents that did not meet contract specifications. This was discovered after termination of the contract by the certification laboratory. These failures have been assigned to one of the two remaining contractors (University of Wisconsin) and that contract has been renewed for an additional year. Therefore, by the end of FY 74 it is anticipated that all reagents will have been produced for phase I and II rhinoviruses.

The certification laboratory remains at the University of Vermont and this laboratory provides data necessary to characterize fully the reagents produced. It is anticipated that this certification contract will continue after eventual termination of production contracts in order to allow completion of work.

RRB is providing low level financial support to a contractor at Ohio State University to determine the extent of phase III rhinovirus serotypes. These results will be discussed with the RRB Advisory Committee at its next meeting and a decision made concerning production of phase III reagents.

Coronaviruses

Two coronaviruses have been selected for reagent production and work is still in progress at the CDC under an interagency agreement. Seed virus preparations have been produced for both types (OC-43 and 229E) and are presently undergoing certification. Control and immune mouse ascitic fluids have also been produced for OC-43 virus. Research and development studies were required for production of antisera against 229E but the appropriate schedule has now been determined for guinea pig immunization and production is in progress.

Adenovirus and Adeno Associated Viruses

Production, testing and packaging of 12 adenovirus seeds and 7 specific antisera which were being remade have been completed during FY 73. The remanufacture of these products was required due to an unexpected loss in titer of seed reagents during storage and excessive cross reactivity of antisera. Release for distribution of these reagents completes the planned activities for this group of viruses. There are now available reagent sets of seed viruses and corresponding antisera for adenovirus types 1 through 31. It is anticipated that, in the future, production activities will be initiated if new additional serologic types are accepted or if more refined reagents are identified which would substantially improve the research capacity of laboratories.

Four AAV types were assigned to North American Biologicals for production and the contractor produced and submitted types 1, 2 and 3; type 4 was not produced due to insurmountable technical problems. Recent results from the certification laboratory indicate that all three seed virus preparations are useful but only two antisera will be suitable reagents and staff will attempt to acquire this antiserum from other sources to complete the set.

Myxoviruses

During FY 73 no seed viruses or corresponding antisera were produced for any of the myxoviruses. The Staff has, however, continued to carry out prior plans

to produce antisera to all the known neuraminidases and hemagglutinins of influenza viruses of man and animals. These reagents will be very useful to laboratories conducting research or epidemiologic studies with this highly mutable virus. At this time production of four of these reagents is completed and they will be packaged shortly and production is nearly completed for two additional types. A contract was recently awarded to the St. Jude Children's Research Hospital for the production of goat antisera to all the remaining types.

The production, testing, and packaging of a small lot of 2-(3'-methoxyphenyl)-N-acetyl-α-neuraminic acid (MPN) has been completed and it is now available for distribution. This synthetic neuraminidase substrate should find wide use in both the qualitative demonstration and quantitative assay of this enzyme. It is expected that MPN will also have application in interferon assays involving influenza viruses. Due to the small quantities available it is planned to initially limit the distribution of this extremely valuable material to collaborative division contractors involved in the influenza programs and intramural scientists before beginning wide-spread distribution to other laboratories.

Hepatitis

This program has now developed a complete set (antisera and antigens) for reference subtyping of hepatitis B subtypes D (ad+ y-) and Y (ay+ d-). These reagents have been distributed to several of the collaborative hepatitis contractors as well as to numerous research laboratories. Both antisera have also been packaged according to WHO specifications. The D antiserum has been evaluated by an international array of laboratories and a manuscript is in preparation to report the results of this cooperative study. When the manuscript is completed staff expects to offer the D antiserum to WHO as an international reference reagent. Preliminary discussions with WHO Staff has indicated that RRB may be able to obtain their assistance in a similar evaluation of the Y antiserum for eventual submission as an international reference.

In cooperation with the AEC-MAN laboratory, antiserum is presently being produced in rabbits for subtype HBAg adr and preliminary results indicate a high titered product with good specificity. It is expected that production of subtype adw antiserum will be subsequently initiated after selection of an appropriate host. Dane particle-rich pools have been prepared from which Dane particles and their cores have been partially purified. Hyperimmune animal serum to core antigens is now being produced in guinea pigs. In collaboration with the intramural staff a solid-phase radioimmunoassay for core antigen has been developed and should be useful for epidemiologic and infectivity studies.

Research Resources Branch in collaboration with Infectious Diseases Branch plans to sponsor a workshop during FY 74 on hepatitis antigens, reagents and methods for laboratory diagnosis.

Herpesviruses

Prior production of these reagents has made available seed viruses and antiserum to herpes simplex type 1, herpesvirus B and pseudorabies viruses. Within the recent past, scientific interest in this group of viruses has grown rapidly and this is reflected in the published literature, papers at scientific meetings and conferences. The Research Resources Advisory Committee, however, felt that insufficient attention has been given to reagents for these viruses. Consequently, Branch Staff has now participated in two planning sessions in preparation for a herpesvirus workshop which is scheduled for the fall of 1973. It is anticipated that leading investigators who are actively working with herpesviruses of human importance will attend and provide a mechanism whereby the need for and feasibility of producing reagents will be identified. Research Resources Branch Staff will use the recommendations of this workshop as a guide to the initiation of a reagent program.

Allergens

In the past years the Branch has acquired and is distributing two small lots of ragweed antigen E prepared for either in vivo or in vitro research and a small lot of ragweed antigen Ra 3 is also available. During FY 73 a contract was negotiated with Worthington Biochemical Corporation to isolate, purify, and package antigens E, K and Ra 3 from 20,000 grams of short ragweed pollen. This contract is progressing on schedule and by its termination in January 1974 these purified antigens will be available for distribution. These are being prepared and packaged for distribution as an Investigational New Drug.

This program has negotiated for the procurement of 10,000 grams of pollen from the 1973 crop of perennial ryegrass (Lolium perenne). Requests for proposal have been issued for extraction, purification and packaging of group I, II and III antigens from this raw product. When completed this will provide allergic disease research laboratories with well characterized antigens some of which were found to be distributed among a variety of grass tribes.

Requests for proposals have also been issued for the production of Asc-1 allergen from Ascaris suum. This extremely potent antigen has the capacity for producing homocytotropic antibodies when administered to laboratory animals. Inclusion of this product into the Branch program will offer material for conducting experimental allergy research.

Proposals have been solicited for the production in rabbits of polyvalent antiserum against the antigens of short ragweed pollen. This product will provide a long term reference for serologic assay of the predominant antigens present in this pollen of well-known importance to allergic disease.

AEC-NIAID Molecular Anatomy Program (MAN)

The Rockville Laboratory of the MAN Program is currently in its fourth year of operation as a cooperative project of the National Institute of Allergy and Infectious Diseases (NIAID) and the U.S. Atomic Energy Commission. The major objectives of this program are the development of methods for isolating viruses and viral antigens and the application of these techniques to research on

associated diseases. The training of NIAID scientists and contractors in the utilization of specialized biophysical instrumentation developed by the MAN Program, particularly the zonal centrifuge, remains an important aspect of this laboratory mission.

Current research at the Rockville Laboratory involves: (1) biochemical, biophysical and immunological characterization of the known subtypes of hepatitis type B (Australia) antigen, (2) preparation of specific hepatitis type B reagents for the Research Resources Branch of the NIAID, (3) development of a purified and inactivated trivalent parainfluenza vaccine, and (4) basic studies with Respiratory Syncytial virus and its ts mutants, the virion associated with nonbacterial gastroenteritis, recombinant influenza viruses, and hepatitis type A virus. Research and development at Oak Ridge in support of this program includes efforts to improve high-speed continuous-flow vaccine centrifuges, to investigate supporting surfaces for radio-immunoassays, and to develop automatic recycling systems for the amplification of separations based on the use of immunoadsorbents. The latter are designed for the isolation of transplantation antigens, autoantigens, viruses and bacterial toxins. This work is broadly concerned with the improvement of methods for detecting and combatting infectious agents.

Processing and Distribution of Reagents

In late FY 72 the contract for processing the reagents distributed by RRB was moved from Ben Venue Laboratories to the American Type Culture Collection in Rockville, Maryland and most of their work has been done in FY 73. This facility is expected to process, lyophilize, package and deliver for distribution units of antigen, immune sera and control sera from approximately 165 reagent lots during a two-year contract period. This contract is a necessary part of the Branch activities and the expertise of the processing laboratory is relied on to prevent degradation of reagents during processing. Due to delays in installation of an adequate lyophilizer and problems related to residual moisture, the lyophilization phases are currently behind schedule. These problems have now been resolved and it is anticipated that the contractor can process the stored reagents with increased speed. Distribution of reagents from the repository at Flow Laboratories follows:

| Fiscal Year | Period of Operation | Total Transactions | Total Amps & Vials Distributed |
|---|---|---|---|
| 1968 | 7/1/67 - 6/30/68 | 391 | 31,776 |
| 1969 | 7/1/68 - 6/30/69 | 402 | 15,805 |
| 1970 | 7/1/69 - 6/30/70 | 474 | 26,242 |
| 1971 | 7/1/70 - 6/30/71 | 638 | 12,902 |
| 1972 | 7/1/71 - 6/30/72 | 572 | 21,801 |
| 1973 | 7/1/72 - 3/31/73 (9 mos) | 460 | 8,673 |

A total of 72,238 ml of bulk has been distributed since the program was initiated. The number of transactions in FY 73 is higher than that of last year at the same time. This increase in shipments can be attributed to the fact that the hepatitis B subtype ay reagents became available in the fall and to the release of the enterovirus typing pools A-H the latter part of the

summer. No large quantities of materials were transferred to other agencies (i.e., Bureau of Biologics or the Center for Disease Control) this year thus reflecting a decrease in the total number of items distributed to date.

Work on a new edition of the Catalog of Research Reagents is behind schedule due to inadequate staffing and other obligations. However, the Branch continues to distribute the 1970 Catalog along with supplemental listings of material not described in the Catalog. Announcements are also placed in various scientific publications when new reagents are released.

3. Contract Narratives

Arbovirus Reagent Program

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH (PH43-68-679)

Title: Production and Testing of Arbovirus Research Reference Reagents

Project Director: Dr. Edwin H. Lennette

Project Officer: Dr. John E. Nutter

Objectives: To produce research reference reagents to St. Louis Encephalitis, Rio Bravo, Yellow Fever (17D), Tembusu, West Nile, Bussuquara, Russian Spring-Summer Encephalitis, Japanese B Encephalitis, Powasson, Semliki Forest, Piry, Mucambo and Venezuelan Equine Encephalitis (Florida).

Major Findings: Production and testing have been completed on Rio Bravo, Yellow Fever, Tembusu, West Nile, and Bussuquara. Production is proceeding on the following reagents: St. Louis Encephalitis, Powasson, Japanese B Encephalitis, Semliki Forest, Russian Spring-Summer Encephalitis, Piry, Mucambo and Venezuelan Equine Encephalitis (Florida).

Significance to NIAID Program and Biomedical Research: This contract will furnish 13 sets of arbovirus reagents to help fill the needs of NIH grantees, intramural scientists, contractors and other eligible recipients of arbovirus reagents.

Proposed Course of Project: This project has been renewed for an additional and probably final year as part of a continuing arbovirus reagent production effort.

Date Contract Initiated: April 22, 1968

Current Annual Level: $63,112

YALE ARBOVIRUS RESEARCH UNIT (PH43-66-460)

Title: Testing and Identification of Arbovirus Reagents

Project Director: Dr. Robert Shope

Project Officer: Dr. John E. Nutter

Objectives: To furnish essential testing and certification services for other arbovirus production contractors.

Major Findings: To date these testing services have been utilized to furnish NIAID Staff and Arbovirus Advisory Committee with information needed to determine acceptability for distribution of arbovirus reagents.

Reports were submitted during the year of tests carried out on 2 bulk pool seed virus reagents, 6 wet-frozen final ampoule seed virus reagents, 6 freeze-dried seed virus reagents, 20 bulk pool immune reagents, and 41 freeze-dried immune reagents. These were tested by HI, CF, and neutralization tests utilizing stocks of the Yale Arbovirus Research Unit. A data storage and retrieval system was used to compile results. In addition, plaque reduction neutralization tests with mouse hyperimmune ascitic fluids of group A viruses indicated specificity of reagents produced in the Research Resources Branch program. Virus seeds of group C have been titrated preparatory to carrying out group C plaque reduction neutralization tests.

Significance to NIAID Program and Biomedical Research: This service is an essential element of the arbovirus reagent program and its full usefulness is now being realized.

Proposed Course of Project: Continuation during arbovirus production and processing efforts.

Date Contract Initiated: January 19, 1966

Current Annual Level: $ 63,952

YALE ARBOVIRUS RESEARCH UNIT (NIH-72-2509)

Title: The Production of Specific Dengue Immune Reagents

Project Director: Dr. Robert Shope

Project Officer: Dr. John E. Nutter

Objectives: To produce and test reagents for Dengue types 2 and 3.

Major Findings: Production is nearly complete on the 250 ml of seed, 300 ml of immune ascitic fluid and the 100 ml of control ascitic fluid.

Significance to NIAID Program and Biomedical Research: This contract has resulted in the production of reagents for Dengue types 2 and 3. These viruses have been exceedingly difficult to work with and these reagents provide reference material for two viruses of significant public health importance.

Proposed Course of Project: Terminated May 1973

Date Contract Initiated: February 1972

Current Annual Level: Prior year support

CENTER FOR DISEASE CONTROL

Title: Catalogue of Arthropod-borne Viruses of the World

Project Director: Dr. Trygve O. Berge

Project Officers: Dr. Earl Chamberlayne and Dr. John E. Nutter

Objectives: To maintain a registry of data derived from investigators on arthropod-borne viruses by cataloging the known physical, chemical and antigenic characteristics, the host and vector range, pathogenesis and geographical distribution of these reagents.

Major Findings: As of June 30, 1972, significant developments were -- change of location of the Catalogue office, progress in revision of the working Catalogue, change in designation and rules of operation of the Catalogue, and formal publication of a second supplement to the first published edition of the Catalogue.

Eight new participants were issued complete Catalogue sets and associated materials (3 in U.S.A., 2 in South America, 1 in Central America, and 2 in Europe). Two participants (U.S.A.) were dropped because of changes in their professional activities. Three assigned Catalogue sets were transferred because of changes in laboratory location (U.S.A. to Brazil, Cambodia to the Ivory Coast, and Taiwan to Switzerland (temporary). At the end of FY 72 (30 June 1972), 153 active sets were maintained in 52 countries. Distribution by continent included: Africa, 13; Asia, 19; Australia, 6; Europe, 29; North and Central America, 69; and South America, 17.

Significance to NIAID Program and Biomedical Research: The continued dissemination of the scientific information pertaining to arboviruses is an essential adjunct to research in arbovirology.

Proposed Course of Project: It is anticipated that this Interagency Agreement will be continued in FY 1974.

Date Contract Initiated: October 1971

Current Annual Level: $26,940

UNIVERSITY OF WEST INDIES (NIH-72-2517)

Title: Research and Development to investigate feasibility of preparing Dengue reagents in rats

Project Director: Dr. Jack L. Price

Project Officer: Dr. John E. Nutter

Objectives: To investigate the feasibility of preparing rat ascitic fluids for Dengue serotypes 1, 3 and 4 by various routes of inoculation and methods of production of immunizing antigen. Preparation of pilot lots of Dengue 2 based on previous experience.

Major Findings: This project should provide definitive information on the production of Dengue reagents in rats. Results reported thus far indicate that the rat is an excellent antibody producer and that adjuvant-induced ascitic fluid from rats is a satisfactory antiserum in all tests.

Significance to NIAID Program and Biomedical Research: The Dengue virus reagents have long been a high priority to the arbovirus program. However, technical problems had not previously permitted production of suitable reagents. Hopefully, methods will be developed in this project to have suitable Dengue reagents prepared in larger quantities.

Proposed Course of Project: To be terminated September 1973

Date Contract Initiated: June 27, 1972

Current Annual Level: Prior year support

UNIVERSITY OF CALIFORNIA (NIH-73-2001)

Title: Production and Evaluation of Antisera Used to Identify Mosquito Blood Meal

Project Director: Dr. Constantine H. Templis

Project Officer: Dr. John E. Nutter

Objectives: To produce and test three antisera used in the identification of mosquito blood meals and to test three additional mosquito blood meal antisera prepared by another contractor.

Major Findings: Chicken anti-Columbiform has been prepared and meets the required criteria specified in the contract. Initial preparation of the anti-Passeriform and anti-Ciconiiform sera has been started. Six lots representing two antisera prepared by another contractor were tested. Only one lot appears to be an acceptable reagent.

Significance to NIAID Program and Biomedical Research: The characterized mosquito blood meal antisera will be a valuable research tool in epidemiological studies of disease transmitted by arthropods by making it possible to identify the blood meal of mosquitoes, phlebotomus flies, etc. important in the transmission of arboviruses.

Proposed Course of Project: To be terminated April 30, 1974

Date Contract Initiated: November 1, 1972

Current Annual Level: $33,338

SMITHSONIAN INSTITUTION (PH43-67-59)

Title: Mammal Identification Service

Project Director: Dr. Charles O. Handley, Jr.

Project Officer: Dr. John E. Nutter

Objectives: Identification of mammalian specimens and processing of collections submitted by NIH and other government affiliated laboratories and institutions.

Major Findings: During the first 9 months of FY 73 this service has provided 1,045 mammal identifications to laboratories. In addition, they published a key to the non-volant mammals of lower Amazonia. This document is unique in that it was prepared without the use of technical terminology. This key will, therefore, be useful for professionally untrained workers identifying mammals from this area of demonstrated importance to arbovirology research.

Significance to NIAID Program and Biomedical Research: This contract supplies an authoritative source for identification of mammalian species as part of studies related to the transmission of infectious diseases and with particular reference to arbovirology.

Proposed Course of Project: Continued support by Research Resources Branch will depend upon availability of funds. At the last renewal the contractor was asked to broaden his base of funding in preparation for a possible reduction in NIAID support.

Date Contract Initiated: December 1, 1971

Current Annual Level: $40,576

Enterovirus Reagent Program

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH (NIH-69-89)

Title: Certification Testing of Enterovirus Reference Reagents

Project Director: Dr. Edwin H. Lennette

Project Officer: Dr. John E. Nutter

Objectives: To perform certification testing of seed virus and antiserum reagents prepared to prototype enteroviruses. In addition, testing of other virus groups will be performed on request.

Major Findings: The certification laboratory confirmed the test data given by the producer or where there have been indications of loss of titer or contamination with other viruses . The contractor has completed the certification testing of both seed and antisera prepared for all enteroviruses that the Research Resources Branch has available for distribution.

Final pools (A-H) of equine enterovirus antisera were tested against the virus types indicated in the scheme, and heterotypic neutralization was seen for certain virus types. The individual antisera responsible for the heterotypic activity were identified, and the pools are being distributed with literature on possible heterotypic neutralization patterns and with individual antisera to permit identification of certain virus types.

Trial pools (J-P) of equine antisera for group A coxsackieviruses were tested against virus types in the scheme, and a few of the viruses were neutralized by heterotypic pools. The individual antisera possessing heterotypic activity were identified, and new pools will be prepared with a revised formula to take into account the heterotypic activity of certain of the antisera.

Enterovirus candidate strain Toluca-1 was shown to have the physicochemical properties of an enterovirus. Enterovirus candidate strain 275/58 from Canada was shown to be immunologically related to coxsackievirus type A7.

Antiserum prepared in ferrets to respiratory syncytial virus strain 18537 had a homologous neutralization titer of 1:128 to 1:320.

Significance to NIAID Program and Biomedical Research: The contractor provides data confirming authentication, identification, potency and purity of enterovirus reference reagents, produced by independent contractors. This comprehensive certification testing enhances the value of the reagents as reference standards.

Proposed Course of Project: As long as a contract is in effect for the preparation of enterovirus reference reagents the services of this laboratory will be needed. The CSDPH and Center for Disease Control will handle future enterovirus activities for the Research Resources Branch.

Date Contract Initiated: December 11, 1971

Current Annual Level: $33,904

CENTER FOR DISEASE CONTROL

Title: Produce seed virus reagents to two picornaviruses and antiserum to one type

Project Director: Dr. Morris T. Suggs

Project Officer: Dr. John E. Nutter

Objectives: To produce three (3) liters each of Coxsackie A-17 (G-12) and enterovirus 68 (Fermon) seed virus reagents, 500 ml of control horse serum and the serum from three horses immunized with enterovirus 68.

Major Findings: Production has been completed and samples have been sent to the certification laboratory for certification of producer's test results.

Significance to NIAID Program and Biomedical Research: The production of these reagents will make available standard reference reagents which are an essential part of those picornaviruses presently in the Research Resources Branch repository.

Proposed Course of Project: Project will be completed when certification results are confirmed.

Date Contract Initiated: January 1972

Current Annual Level: Prior year support

Adenovirus Reagent Program

FLOW LABORATORIES (NIH-71-2288)

Title: Production of Adenovirus Reagents

Project Director: Dr. Jerome Kern

Project Officers: Dr. Malcolm Hoggan and Dr. John E. Nutter

Objectives: To produce, test and package AAV free seed virus reagents to twelve adenovirus types and antisera to seven adenovirus types.

Major Findings: Production has been completed as follows: Adenovirus types 8, 9, 13, 15, 16, 17, 18, 22, 23, 24, 26 and 27 seed virus and Adenovirus types 2, 4, 8, 9, 10, 13 and 14 rabbit antisera.

Significance to NIAID Program and Biomedical Research: These reagents are essential in biomedical research due to their significant role in diseases of man. This virus group has been associated, along with the rhinovirus and myxovirus group, with cold like respiratory diseases.

Proposed Course of Project: Project was completed January 1973.

Date Contract Initiated: June 1971

Current Annual Level: Prior year support

CENTER FOR DISEASE CONTROL

Title: Certification of Adenovirus Reagents

Project Director: Dr. Walter Dowdle

Project Officer: Dr. John E. Nutter

Objectives: Perform laboratory tests required to certify the seed viruses to twelve adenovirus types and antisera to seven adenovirus types.

Major Findings: Potency testing was completed for twelve (12) bulk adenovirus seed lots. Complete homologous and heterologous testing was performed on seven (7) bulk antiserum lots.

Significance to NIAID Program and Biomedical Research: These reagents are essential in biomedical research due to their significant role in diseases of man. This virus group has been associated, along with the rhinovirus group, with cold like respiratory diseases.

Proposed Course of Project: Project is complete

Date Contract Initiated: July 1971

Current Annual Level: Prior year support

NORTH AMERICAN BIOLOGICALS, INC. (NIH-71-2100)

Title: Production of Adeno-Associated Virus Reagents

Project Director: Dr. Robert R. Rafajko

Project Officers: Dr. James Rose and Dr. John E. Nutter

Objectives: To produce, test and package seed virus reagent and antisera to four adeno-associated virus reagents.

Major Findings: Production has been completed and results obtained to date indicate that seed reagents for types 1, 2 and 3 and the antisera for types 2 and 3 will be useful as reagents.

Significance to NIAID Program and Biomedical Research: This contract furnished adeno-associated virus reagents which are an essential part of the total adenovirus reagent program due to their adventitious nature in adenovirus seeds.

Proposed Course of Project: Terminated February 1973

Date Contract Initiated: April 6, 1971

Current Annual Level: Prior year support

WORTHINGTON BIOCHEMICAL CORPORATION (NIAID-73-2507)

Title: Extraction of Antigens E, Ra 3 and K from Low Ragweed Pollen

Project Director: Dr. Robert Flora

Project Officer: Dr. John E. Nutter

Objectives: To extract, process, and package purified antigens E, K and Ra 3 suitable for in vivo and in vitro immunological studies.

Major Findings: Production of the pilot lots is nearing completion. It is expected that samples of this pilot project will be submitted to the certification laboratories within a few weeks. Work on the remaining bulk pollen will not be initiated until the results have been reported and reviewed for the pilot batch.

Significance to NIAID Programs and Biomedical Research: Of the thirteen ragweed antigens, only three have been fully characterized; antigen E, antigen K and antigen Ra 3. Earlier production contracts resulted in pilot lots of antigens E and Ra 3 but the present Worthington contract will result in large lots of each of the 3 antigens. These will serve as valuable reference standards and the antigens will be packaged for use in human clinical research.

Proposed Course of Project: To be completed by January 1974

Date Contract Initiated: October 1972

Current Annual Level: $85,000

Rhinovirus Reagent Program

ABBOTT LABORATORIES (NIH-71-2114)

Title: The Production and Testing of Rhinovirus Research Reference Reagents

Project Director: Mr. George S. Marquis

Project Officer: Dr. Albert Z. Kapikian, Dr. John E. Nutter

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to 20 prototype rhinoviruses.

Major Findings: Of the twenty seed virus reagents prepared, 18 have been packaged and sent to the repository; two more are in bulk awaiting approval to package. Production activities have been completed for all the antiserum types.

Significance to NIAID Program in Biomedical Research: These reagents are essential in biomedical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups, with cold like respiratory diseases.

Proposed Course of Project: To be terminated June 1973

Date Contract Initiated: April 1971

Current Annual Level: Prior year support

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH (NIH-71-2112)

Title: The production and testing of Rhinovirus Research Reference Reagents

Project Director: Dr. Jack Schieble

Project Officers: Dr. Albert Z. Kapikian and Dr. John E. Nutter

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to five prototype rhinoviruses.

Major Findings: Production of antigen and antiserum for five rhinovirus types has been completed. Samples of the bulk material are now being tested by the certification laboratory.

Significance to NIAID Program and Biomedical Research: These reagents are essential in biomedical research due to their significant role in diseases of man. This virus group has been associated along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: Terminated April 1973

Date Contract Initiated: March 1971

Current Annual Level: Prior year support

UNIVERSITY OF WISCONSIN (NIH-71-2111)

Title: The production and testing of Rhinovirus Research Reference Reagents

Project Director: Dr. Elliot Dick

Project Officers: Dr. Albert Z. Kapikian and Dr. John E. Nutter

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to ten prototype rhinoviruses.

Major Findings: Five bulk seed and antiserum types have been prepared and sent to the repository. Samples of bulk material have been tested for four additional sets. All materials from this contract have substantially exceeded the contract specifications. Rhinovirus type 65 presented some problems, and reagents for this type will be remade.

Significance to NIAID Program and Biomedical Research: These reagents are essential in biomedical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be continued for an additional year to remake 5 rhinoviruses (4 are failures by another contractor).

Date Contract Initiated: April 25, 1971

Current Annual Level: $7,600 (3 month extension)
$23,866 (production of five rhinoviruses)

SOUTHWEST FOUNDATION FOR RESEARCH AND EDUCATION (NIH-71-2110)

Title: The production and testing of Rhinovirus Research Reference Reagents

Project Director: Dr. S. S. Kalter

Project Officers: Dr. Albert Z. Kapikian and Dr. John E. Nutter

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to six prototype rhinoviruses.

Major Findings: Production was completed for six antigens and antiserum types. However, certification results indicate that four of the seeds do not meet contract specifications and will have to be remade by another contractor.

Significance to NIAID Program and Biomedical Research: These reagents are essential in biomedical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: Terminated December 1972

Date Contract Initiated: April 1971

Current Annual Level: $5,856 (5 month extension)
$4,125 (adjust overhead)

UNIVERSITY OF VIRGINIA (NIH-71-2113)

Title: The production and testing of Rhinovirus Research Reference Reagents

Project Director: Dr. Jack Gwaltney

Project Officer: Dr. Albert Z. Kapikian

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to five prototype rhinoviruses.

Major Findings: Production of antigen and antiserum for six (6) rhinovirus types was completed. Results obtained thus far from the certification laboratory indicate that all are of satisfactory potency and purity.

Significance to NIAID Program and Biomedical Research: These reagents are essential in biomedical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: Terminated July 1972

Date Contract Initiated: April 1971

Current Annual Level: Prior year support

UNIVERSITY OF VERMONT (PH43-67-1188)

Title: To test, identify and certify Rhinovirus Reference Reagents

Project Director: Dr. Charles A. Phillips

Project Officers: Dr. Albert Z. Kapikian and Dr. John E. Nutter

Objectives: To authenticate fifty-six Phase I rhinovirus reagents produced by Abbott Laboratories for identity, potency and purity. To authenticate Phase II rhinovirus reagents and any Phase I remakes to be produced by five production contractors.

Major Findings: Phase I testing - 56 seed viruses in final vials, 56 aliquots of bulk sera pools and 56 sera in final vials have been received for testing. All antigens, including nine which were remade, seem acceptable. All 56 bulk sera have acceptable titers. Phase II testing - 22 antigens and 20 antisera (bulk) have been tested and 6 additional antigens and antisera are currently under test.

Significance to NIAID Program and Biomedical Research: The contract furnishes authentication and purity confirmation on rhinovirus reagents being or to be produced by other independent contractors. The comprehensive certification testing will enhance the value of the rhinovirus reagents as reference standards.

Proposed Course of Project: The services of this contractor will be required until the Phase II rhinovirus production has been completed.

Date Contract Initiated: June 1967

Current Annual Level: $51,415

Myxovirus Reagent Program

ST. JUDE CHILDREN'S RESEARCH HOSPITAL (NIH-72-2510)

Title: Research and Development in Production of monospecific influenza antisera

Project Director: Dr. Robert G. Webster

Project Officer: Mr. Robert M. Pennington

Objectives: To perform research and development studies to provide the methodology for preparing "monospecific" antisera to the hemagglutinin subunits of influenza viruses such as PR8 and FM1 where the hemagglutinin subunits are unstable in sodium dodecyl sulphate (SDS).

Major Findings: Hemagglutinin subunits were isolated by electrophoresis from the reference strains of HON1 (PR8) and H1N1 (FM1) influenza viruses after treatment of the virions with sodium deoxycholate and Tween 20. The isolated subunits were short rods measuring 12 x 4 nm and contained two polypeptide chains of approximately 55,000 and 20,000 molecular weight. Rabbits were immunized with the isolated subunits, and chickens were immunized with the isolated subunits, and chickens were immunized with DOC treated virus absorbed to autogeneic erythrocytes. Sera prepared in these ways contained antibodies to the hemagglutinin subunit but no detectable antibodies to the other virus proteins.

Significance to NIAID Program and Biomedical Research: Antisera to the isolated hemagglutinins and neuraminidases of type A influenza viruses will provide researchers and epidemiologists with a non-infectious reference standard for studying the surface antigens of influenza viruses of human, equine, porcine and avian origin. These reagents will enable investigators to study the inter-relationships among viruses from human and non-human sources and will also facilitate studies on antigenic shifts which characterize influenza virus and play a key role in epidemic disease.

Proposed Course of Project: Terminated September 1972

Date Contract Initiated: March 8, 1972

Current Annual Level: Prior year support

ST. JUDE CHILDREN'S RESEARCH HOSPITAL (AI 32516)

Title: Preparation of Antisera to Isolated H and N Subunits of Influenza Viruses

Project Director: Dr. Robert G. Webster

Project Officer: Dr. John E. Nutter

Objectives: To produce 2-3 liters of goat antisera to the isolated hemagglutinins of A/PR/8 and A/FM/1/47 and to perform research and developmental studies to determine the technical feasibility before proceeding with the production activities for 2-3 liters of goat antisera to the known neuraminidases and hemagglutinins of the influenza viruses of man and animals.

Major Findings: None to date as project was initiated recently.

Significance to NIAID Program and Biomedical Research: Antisera to the isolated hemagglutinins and neuraminidases of influenza viruses will provide researchers and epidemiologists with a non-infectious reference standard for studying the surface antigens of influenza viruses of human, equine, porcine and avian origin. These reagents will enable investigators to study the inter-relationships among viruses from human and non-human sources and will also facilitate studies on antigenic shifts which characterize the influenza viruses and play a key role in epidemic disease.

Proposed Course of Project: At least two years will be required to complete this project.

Date Contract Initiated: March 22, 1973

Current Annual Level: $44,676

MOUNT SINAI SCHOOL OF MEDICINE (NIH-72-2511)

Title: Production of MPN, a synthetic neuraminidase substrate

Project Director: Dr. Peter Palese

Project Officer: Mr. Robert M. Pennington

Objectives: To produce 5 grams of neuraminidase substrate 2-(3'methoxyphenyl)-N-acetyl-α-neuraminic acid (MPN)

Major Findings: Material was produced as required; packaged by another contractor and has now been placed in the Research Resources repository for distribution.

Significance to NIAID Program and Biomedical Research: The MPN will provide useful information in demonstration of neuraminidase and will also have application in interferon assays involving influenza viruses.

Proposed Course of Project: Terminated September 1972

Date Contract Initiated: March 1972

Current Annual Level: Prior year support

## Zonal Centrifuge Program

AEC-NIAID

Title: Molecular Anatomy (MAN) Program (Rockville and ORNL)

Project Director: Dr. Norman Anderson (Director, ORNL)

Project Investigator: Dr. John Gerin (Resident Director, Rockville)

Project Officers: Dr. Robert J. Byrne, Dr. George Galasso and Dr. John E. Nutter

Objectives: MAN (ORNL) - To develop biophysical and engineering methodology applicable to NIAID Programs. MAN (Rockville) - 1) to conduct collaborative research with NIAID Intramural scientists and collaborative research contractors; 2) to apply separation systems developed through MAN programs at Oak Ridge; 3) to train NIAID intramural scientists and collaborative research contractors in separation techniques.

Major Findings: Highly purified 20 nm forms of each available subtype of HBAg contain essentially identical polypeptides which differ not in size but in their relative proportions. Available data indicates that the renatured polypeptides possess both group (a) and type (dy) specific antigenic determinants which are dependent upon intrapeptide disulfide bonds. Amino acid analysis of the native particulate ayw and adw antigens revealed no difference. Antiserum is currently being produced to purified HBAg adr and the data indicates that this rabbit antiserum will be useful as a subtyping reagent for the r determinant.

Dane particle-rich pools have been prepared from which Dane particles and their cores have been partially purified. Hyperimmune animal serum to core antigens is now being produced in guinea pigs in the animal facility. In collaboration with Dr. R. Purcell (NIAID) a solid-phase radioimmunoassay for core antigen has been developed which should be of great importance in epidemiologic and infectivity studies. No morphological form of HBAg contains reverse transcriptase enzyme activity, however, independent collaboration with Dr. W. Robinson (Stanford) and Dr. B. Gerwin (NCI) revealed an association of DNA-dependent DNA polymerase enzyme activity with the Dane particles. Preliminary experiments indicate an association of the polymerase activity with the 27 nm core of the Dane particle. If fully substantiated, this finding is of importance since it will establish the Dane particle as the virus of type B hepatitis possessing a DNA genome.

A zonal centrifuge purified and concentrated parainfluenza 3 experimental vaccine was prepared which produced high levels of neutralizing antibody in hamsters; the vaccine is presently on accelerated stability testing and its immunogenicity was fully stable after one week incubation at 37°C.

Biophysical characterization of a virion associated with nonbacterial gastroenteritis revealed properties consistent with those of the parvoviruses.

Further characterization and isolation of the virus has been actively pursued, and hyperimmune serum to the purified virus was produced in guinea pigs. A specific and quantitative serologic assay is currently under development.

Basic studies on the antigenic structure and biochemical properties of RS virus continued in FY 1973. The surface projections, glycoprotein spikes, of RS were isolated from purified virus and animal antiserum to the spike possessed virus-specific complement-fixing antibody.

A number of NIAID fellows have received training at the Rockville Laboratory as part of their research programs. In addition Dr. T. S. Thornhill (NIAID) has been assigned to the laboratory as a guest worker on the nonbacterial gastroenteritis project and Mr. Ami Vonsover (Israel) is spending six months with us as a WHO fellow.

Significance to NIAID Program and Biomedical Research: The development and utilization of these systems provides a unique opportunity for the purification of viruses and virus subunits which will have application in the development of more effective vaccines and reagents. The program also affords the opportunity for training of NIAID and Collaborative contract scientists.

Date Contract Initiated: April 24, 1964

Current Annual Level: $475,000

CENTER FOR DISEASE CONTROL

Title: Production of Coronavirus Research Reagents

Project Director: Dr. Morris T. Suggs

Project Officer: Dr. Albert Kapikian

Objectives: Prepare research reference reagents against two strains of coronaviruses (OC 43 and 229E)

Major Findings: Two lots of seed virus reagent for OC 43 have been prepared and samples have been sent for certification. Immune serum for this type has been prepared and is now being tested by the producer. Seed virus for 229E has been prepared and samples have been sent to the certification laboratory. Production for antiserum to this type is still in progress.

Significance to NIAID Program and Biomedical Research: Since 16% of the lower respiratory tract infections are known to be caused by these two coronavirus strains reference reagents prepared against OC 43 and 229E would be very useful to the scientific community.

Proposed Course of Project: To be completed within FY 74

Date Contract Initiated: July 1971

Current Annual Level: Prior year support

## 4. PUBLICATIONS

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INFECTIOUS DISEASE BRANCH

Summary

The Infectious Disease Branch (IDB) of Collaborative Research sponsors research projects which are directed toward: (1) control of infectious diseases that are particularly prevalent in the U.S. population or that seriously affect designated segments of the population, and (2) improvement of already existing control measures for certain diseases. A majority of projects supported by the IDB are those for which the research tools and techniques are available and can be applied through directed contract funding support. A limited number of basic studies fill critical scientific gaps in existing programs.

The activities of the IDB are divided into four broad programs: (1) Viral Vaccine Development, (2) Bacterial Vaccine Development, (3) Hepatitis, and (4) Antiviral Substances. Three of these four programs are strongly related to the NIAID's intramural research programs

The Viral Vaccine Program sponsors research for the control of infections caused by respiratory syncytial virus (RSV), parainfluenza viruses, adenoviruses, influenza viruses, smallpox and rubella virus.

During the past year, clinical trials have emphasized testing of potential temperature sensitive RSV vaccines in children and live attenuated influenza vaccine in adults. These clinical studies will be continued in fiscal year 1974 and, in addition, it is expected that an inactivated parainfluenza vaccine will be ready for testing. The NIAID is completing its survey to determine a smallpox vaccination procedure which would result in reduced morbidity; these data will be valuable if future smallpox outbreaks occur, as have been seen recently in Yugoslavia and England. Studies on the subcutaneous route of inoculation may also be of value in determining the proper route of vaccination for eczematous children.

The Bacterial Vaccine Program sponsors research for the control of infections caused by pneumococci, meningococci, Hemophilus influenzae, streptococci, and mycoplasma.

During the past year, clinical trials have been conducted with candidate H. influenzae vaccine and Neisseria meningitidis vaccine in order to develop vaccines that could be used to reduce the incidence of bacterial meningitis in young children. The pneumococcal vaccine program is directed toward developing a Streptococcus pneumoniae vaccine for high-risk populations such as elderly persons and others with debilitating diseases.

Pneumococcal vaccine is being evaluated at several centers, and current evidence indicates that this vaccine is effective and could be used in a large field trial.

Inactivated Mycoplasma pneumoniae vaccine has been prepared for eventual use in high-risk populations such as military recruits and young adults, as well

as the pediatric population. The bacterial vaccine program also includes the development of a group A streptococcal vaccine for use in preventing strep throats and concomitant illnesses.

The IDB sponsors a number of vaccine evaluation centers which provide facilities for Phase 1 and Phase 2 testing of vaccine candidates. These centers include facilities for closed populations wherein patients can be isolated singly or in small groups when live agent vaccines are used; they also provide facilities for testing in larger groups ranging from day care centers to old age centers and from chronic disease centers to group health centers. This enables the IDB to test all manner of candidate vaccines in a variety of populations.

In September 1971 the hepatitis program was initiated as a major effort of the Infectious Disease Branch. The goal of this program is to develop active and passive immunization procedures for hepatitis A and B. The Institute has carefully planned the approach to achieving control methods for hepatitis B, and all current activities of the Branch are directed toward this end. During the past year progress has been made in developing the chimpanzee and rhesus monkeys as hepatitis B animal models. The hepatitis B agent has been characterized in the rhesus monkey as to its heat sensitivity, routes by which the animal becomes infected, and information is being gathered on the infectious unit. Animal models will be essential in the screening of experimental vaccines. Clinical studies on passive immunization are now under way to determine if hepatitis B antibody gamma globulin can prevent hepatitis B. In fiscal year 1973 the hepatitis program has initiated contracts to gather data on the epidemiology of hepatitis B in populations in which hepatitis B is endemic. All NIAID collaborative hepatitis studies are furnished with services from the Hepatitis Testing Laboratory which was initially funded in FY 1972. This laboratory provides hepatitis B antigen and antibody testing.

The Antiviral Substances Program (ASP) was initiated in fiscal year 1970 to develop prophylactic and therapeutic methods for the prevention and treatment of virus diseases which are serious public health problems.

The ASP continues to pursue its efforts directed toward determining the clinical usefulness of antiviral substances, particularly interferon. This includes the development of interferon assays, preparation of reference reagents for interferon research, development of methods to produce large amounts of interferon, and development of purification procedures. Interferon has successfully been produced in human leucocytes and diploid cells, and this and other interferon preparations are being tested against human diseases and in animal models. During the past year the IDB has launched several clinical studies to determine the effectiveness of interferon treatment in preventing various herpetic infections. Other clinical studies include efforts to determine the effectiveness of 9-B-D arabinofuranosyl adenine and iododeoxyuridine to prevent herpes encephalitis and other serious viral diseases.

The research sponsored by this program continues in its directed effort to attempt to bridge the gap from scientist-originated research to practical use of this information in man.

## Program Status Report

The IDB underwent extensive reorganization of the vaccine evaluation centers in fiscal year 1972, and these changes were solidified during FY 1973 and proved to be extremely beneficial to the total program. Efforts have been concentrated in live respiratory syncytial and influenza virus vaccines; work with bacterial vaccines was concentrated on pneumococcal, Hemophilus influenzae b and meningococcal polysaccharides. Particular emphasis was placed on determining an eventual pediatric dosage for a meningitis vaccine composed of these polysaccharides. The Antiviral Substances Program initiated clinical trials using exogenous interferon, interferon inducers, and the antiviral compounds adenine arabinoside (Ara-A) and iododeoxyuridine (IDU). The Hepatitis Program is now well underway with interrelated contracts in animal models, epidemiology, etiology and clinical evaluation of hyperimmune gamma globulin.

### VACCINE DEVELOPMENT AND VACCINE EVALUATION CENTERS

The goal of the vaccine program is to develop vaccines for prevention of serious infectious diseases for which adequate vaccines are not now available. Rutgers University received a contract for the development of live virus mutants to respiratory syncytial virus (RSV) and parainfluenza virus types 1, 2 and 3 for use as vaccines. Flow Laboratories continues to prepare and safety test the vaccines developed by Laboratory of Infectious Diseases (LID) scientists and eventually those developed by Rutgers. In addition, Flow Laboratories, in collaboration with the Atomic Energy Commission Molecular Anatomy (MAN) Laboratory program, is preparing inactivated parainfluenza virus vaccines. About 20,000 doses of inactivated Mycoplasma pneumoniae vaccine prepared under contract by Ohio State University are available for clinical evaluation. Two lots of inactivated influenza virus vaccine totaling 60,000 doses will be purchased from Lederle Laboratories for IDB-sponsored studies.

The IDB has emphasized two kinds of vaccine evaluation centers: general study centers where adults and children (either free-living or closed populations) are available, and the principal investigator has interest and expertise in the broad area of vaccine evaluation, e.g., University of Colorado, Vanderbilt University, and University of Rochester; and special study centers where only one type of population is available and/or the principal investigator only has primary interest in evaluating a particular type of vaccine, e.g., University of Southern California and University of Connecticut.

### VIRAL VACCINE PROGRAM

Respiratory Syncytial Virus (RSV) - Parainfluenza Viruses: Respiratory syncytial and parainfluenza viruses are the principal causes of severe lower respiratory tract disease among infants and children. The need for effective vaccines to reduce the morbidity and economic toll of these pediatric viral illnesses remains apparent. A temperature-sensitive (ts) mutant of respiratory syncytial virus, developed by the Laboratory of Infectious Diseases, NIAID, is now being evaluated in infants of one year of age.

During the past year, several centers tested different dosages of RSV ts-1 in young children. Since higher concentrations of this vaccine resulted in

some adverse reactivity, the dose has been reduced. The results to date from the reduced dosages are not conclusive since some illness was observed both in the vaccinated and the control groups. No virus spread to susceptible contacts nor reversion of the shed virus to the wild type temperature sensitivity pattern was observed.

A new lot of vaccine, RSV ts-7, is being produced at Flow Laboratories and will be available for clinical evaluation within the next few months. Dr. Simpson at Rutgers is developing mutants to RSV and parainfluenza virus types 1, 2 and 3 for use as live virus vaccines. None of his candidate vaccine viruses will be available for clinical evaluation this year.

A recently purified concentrated lot of inactivated parainfluenza virus type 3 virus has been shown to be very antigenic and stable as measured by serum neutralizing antibody in accelerated stability assays in hamsters. Additional studies with parainfluenza virus grown in eggs are planned. This vaccine will probably be available for clinical evaluation in FY 1974.

Influenza Viruses: During the past year the major thrust has been on the live attenuated influenza A virus ($H_3N_2$) ts-1 (E) vaccine developed by the NIAID's Laboratory of Infectious Diseases. This vaccine has been tested in about 200 young adults with no evidence of illness and appears promising for expanded studies to demonstrate efficacy in the elderly and in young children.

The NIAID Influenza Steering Group held two workshops during FY 73 on the epidemiology of influenza and on influenza vaccines. The group is addressing itself to the possible pandemic of the late 1970's, with a view toward recommendations for research activities leading to aborting or lessening the effects of future pandemics.

Rubella: Investigations of the epidemiology, clinical characteristics and viral immunology of rubella, congenital rubella and rubella vaccines are continuing under one longitudinal study. Although safety of vaccines for individual recipients and their contacts is now established, potential hazards to the fetus by inadvertent vaccination of the mother during pregnancy and efficacy of rubella vaccine-induced immunity are crucial areas requiring continued study.

Smallpox: In FY 1971 the NIAID initiated a collaborative study to try to determine a smallpox vaccine and/or vaccination procedure that would result in reduced morbidity as compared to the current procedure.

Four different vaccines were administered by the percutaneous route at the standard concentration and one and two 10-fold dilutions less. Antibody response as measured by the hemagglutination assay was similar for three of the vaccines at all three concentrations. The fourth vaccine did not appear to be as antigenic as the other three. Standardized follow-up studies, including revaccination, are being completed.

The four vaccines are also antigenic when administered subcutaneously at three different lower concentrations. Some of the recipients have had

swollen arms and/or erythema at the site of injection, but from preliminary data it appears that fewer complications occur when the vaccines are given subcutaneously.

While these studies are not expected to provide any data on the more serious complications, such as post-vaccinial encephalitis, they are providing valuable data which should enable the IDB to disseminate the information to smallpox control authorities. A method which carries less risk of some of the complications encountered with the present vaccines and methods of vaccination would be particularly applicable to those countries now free of smallpox.

Data are still being generated by the collaborators in this study, and it is expected that it will take about six months for the study data to be compiled. At that time the staff and collaborators will analyze the data as a whole to determine the best vaccination procedure.

Adenovirus: Live oral adenovirus type 1, 2, 5, 14 and 21 vaccines have been clinically evaluated in adult volunteers. Each of these five vaccines induces a silent infection of the gastrointestinal tract with the development of serum antibody. Although adenovirus type 3 vaccine is now available for evaluation in adults, no studies are presently contemplated. A decision of whether to evaluate adenovirus type 1, 2 and 5 vaccines in children, the target population for these "pediatric" adenovirus types, will not be made until a pediatric formulation is obtained and until studies with crystalline subunit vaccines have been completed.

Crystalline adenovirus type 5 hexon and fiber antigen vaccine was prepared through a collaborative effort of Dr. Julius Kasel, Laboratory of Clinical Investigation, NIAID, and Dr. H. G. Pereira, Mill Hill, England. Both vaccines have been shown to be antigenic and safe in adults. At the present time they are being clinically evaluated in young teenagers.

Rhinoviruses: At the completion of phase 3 of the rhinovirus typing program, the program will terminate. Approximately 110 serologically distinct rhinoviruses will have been identified by that time. With the exception of certain military bases and possibly some other small defined populations, there is no preponderance of a limited number of rhinovirus serotypes associated with respiratory disease so a rhinovirus vaccine is not feasible.

BACTERIAL VACCINE PROGRAM

Bacterial Meningitis: Two organisms (namely Hemophilus influenzae and Neisseria meningitidis) are primarily responsible for bacterial meningitis of young children and infants. Susceptibility to septic infections with these two bacterial agents is exceedingly high in children between 6 and 24 months of age. The precise incidence of meningitis caused by H. influenzae in the United States is not available. However, indirect calculations provide an estimate of the problem as 12,000 infants and young children having had H. influenzae meningitis in 1971. With a mortality rate of 5 percent, approximately 600 children died, and 3,600 children were permanently maimed by neurological defects during this period.

Annually at least 10,000 cases of bacterial meningitis are caused by meningococci (N. meningitidis). Since 90 percent of the meningitis cases occur in infants and toddler children, it is apparent that this segment of the pediatric population would benefit by immunoprophylaxis. Modern technology in immunochemistry has led to purified capsular polysaccharides which appear to be specific immunogens against these bacterial agents.

The candidate H. influenzae vaccine under study by the Infectious Disease Branch has been developed and tested by Drs. Anderson and Smith at the Children's Hospital Medical Center of Boston. Through the contract mechanism, this academic center not only performs clinical evaluation studies but also serves as a central serological testing laboratory for quantitating antibody response by a highly sensitive radioimmunoassay (RIA).

Studies are in progress to determine the protective role of non-polyribose phosphate (PRP) surface antigens (NPS-Ag) of H. influenzae.

During the past year, the IDB has made good progress in its program of clinical evaluation of the Walter Reed Army Institute of Research (WRAIR) meningococcal polysaccharide vaccines (groups A and C) in children and infants. The contract with the Rockefeller University to serve as a central testing laboratory for the radio-antigen binding (RAB) test has made it possible to process over 3,000 serum antibody determinations to both group A and group C meningococcal antigens. Satisfactory progress toward the development of an RAB test to quantitate serum antibody to group B polysaccharides has been made during the past year.

Through a contract with the University of Connecticut, considerable insight has been gained on the immune response of young infants to group A and C meningococcal polysaccharide vaccines. Studies in infants have shown group A and group C vaccines to be safe, with no significant short-term adverse effects. Based on preliminary RAB data, very few infants at 3 months of age have antibody against meningococcal group C polysaccharide (pre-immune mean levels were 0.11 µg or less). Following immunization with 25 or 100 µg of group C vaccine, the majority of infants respond with a mean antibody concentration of 0.75 µg/ml. If a booster dose is given at 7 months, there is no significant rise in antibody level. This observation indicates that infants of this age have made their maximal response to these two dosages of group C polysaccharide.

Pneumococcal Vaccine Program: During FY 1973, extensive work has been done with a radioimmunoassay based upon a modification of the Farr technique, and certain advantages of the RIA test have been realized. Studies have emphasized that reliance on serological confirmation of the diagnosis of pneumococcal disease of the lower respiratory tract is a necessary adjuvant to accurate epidemiologic investigation.

The radioimmunoassay of pneumococcal antibodies correlates well with the quantitative precipitin and mouse protection tests. Further, the RIA can be adapted to measure capsular polysaccharides and antigen-antibody complexes in serum and can be used to assess the purity of pneumococcal vaccines. To date, phase I clinical evaluation has included 954 immunizations of

volunteers with monovalent and polyvalent vaccines of pneumococcal capsular polysaccharides prepared under contract by Eli Lilly and Company. Four hundred fifty-two of these immunizations have been carried out during the first nine months of FY 1973. One dodecavalent and two hexavalent vaccines have been investigated for reactivity and antigenicity.

In January 1973 a double-blind controlled vaccine trial with two hexavalent vaccines was initiated at the Dorothea Dix Hospital, Raleigh, North Carolina. More than 460 volunteers have been enrolled in the study. It is estimated that by July 1, 1973, 850 individuals will have entered the study.

Based on the data generated by a feasibility study at Kaiser Permanente Medical Group of San Francisco and recommendations made by a project site visit team, there appears to be ample evidence to suggest a controlled vaccine trial could be staged in this study center in early FY 1974.

During the past year, the Infectious Disease Branch conducted two workshops devoted to bacterial polysaccharide vaccines: (1) "Immune Response to Meningococcal, Pneumococcal and Hemophilus Influenzae Type b Polysaccharides" (NIH, December 4-5, 1972) and (2) "New Approaches for Inducing Natural Immunity to Pyogenic Organisms" (jointly sponsored by National Institute of Child Health and Human Development at Winter Park, Florida, March 21-23, 1973).

Streptococcal M-Protein Vaccine: Group A streptococcal infections play a major part in septic pharyngitis and skin infections as well as an antecedent role in rheumatic fever and acute glomerulonephritis. An effective multivalent vaccine containing at least six of the predominant serotypes of M-protein would make a major impact on reducing the prevalence of these serious illnesses.

The contract with the University of Chicago has led to the development of several satisfactory candidate M-protein vaccines which currently are being evaluated in children and adult volunteers for safety and immunogenicity. Studies completed during the last year include immunization of 19 adults by subcutaneous injection and 16 by intranasal application. These 35 vaccinees were challenged with live type 1 streptococci along with 40 control subjects. In addition, 30 children have been immunized by the intranasal route without challenge. All vaccinees were studied for serum antibody responses by three methods: passive hemagglutination assay, complement fixation, and bactericidal assay.

To encourage the exploration of other methodology to prepare pure streptococcus group A M-protein antigens, qualified sources were sought to prepare at least 2.0 gm of crude M-protein each of serotypes 1, 6 and 12. Upon completion, this crude M-protein will be divided and distributed to competent investigators desiring to collaborate in the program. These investigators will conduct studies on the purification and characterization of potency and immunogenicity of these preparations.

Mycoplasma Pneumoniae Vaccine: As part of a collaborative program, a total of 20,000 doses of inactivated M. pneumoniae vaccine have been prepared and safety tested by Ohio State University and Cornell University. In

antigen extinction tests in animals, these vaccines appear to be 10 times as antigenic as the best previous lot. The Laboratory of Infectious Diseases immunized hamsters intranasally, then challenged them with wild type M. pneumoniae and found that they were protected; 13/13 vaccinees at Ohio State University seroconverted as measured by the complement fixation (CF) test using whole cell or lipid antigen.

Three other studies are planned in adults: one will include a challenge study in volunteers immunized intramuscularly; a second study will immunize volunteers intramuscularly or intranasally. If appreciable local secretory antibody is formed following intranasal administration of vaccine, challenge studies will be performed. A third study will involve immunizing volunteers either intramuscularly, intranasally, or intramuscularly and intranasally combined. Local and serum antibody responses will be measured. If the vaccines appear antigenic and protective, a field trial in military recruits will be designed.

HEPATITIS

The NIAID Collaborative Hepatitis Research Program has as its long-range goal research activities leading to control of both hepatitis A and hepatitis B infection. Major emphasis is now being placed on a study of hepatitis B infection, since recent knowledge of hepatitis B antigen and antibody characteristics has made it feasible to better define this disease. The following is a description of the progress. The projects were initiated after careful Institute planning which included a series of workshops on hepatitis B subtypes, animal models, immune complexes and hepatitis, immunization and epidemiology.

Animal Models: Progress has been made in characterizing the agent of hepatitis B in animal models. The general susceptibility of primates to hepatitis B, as recognized by development of antibody to HB Ag, appears to parallel the phylogenetic scale, with those primates closest to man being the most susceptible, notably the chimpanzee and the rhesus monkey.

A pool of hepatitis B (subtype adw) has been established in the rhesus monkey, and the characteristics of this pool are now being determined such as heat sensitivity, chloroform and formalin inactivation data, and determination of infectious route, infectious unit and antibody response. Current studies indicate that the hepatitis B agent can be inactivated and still produce an antibody rise. A known human infectious pool of hepatitis B serum is being titrated in the chimpanzee in preparation for future vaccine trials.

Testing Laboratory: One of the problems in hepatitis B studies is that it is difficult to get accurate and consistent test results for HB Ag and HB Ab. During this past fiscal year the NIAID has developed the Testing Laboratory at the American Red Cross (ARC). The Laboratory's function is to test for hepatitis antigens and antibodies by the most sensitive techniques available. Tests currently available include subtype identification, radioimmunoassay procedures for antigen and antibody, and passive hemagglutination tests. During the year the Laboratory has performed hepatitis B antigen and antibody tests for the NIAID hepatitis programs and also for the hepatitis programs of

other NIH Institutes and the Food and Drug Administration. An additional function of the Testing Laboratory is to aid in the standardization of methods for detecting hepatitis antigens and antibodies.

Epidemiology: The NIAID, in collaboration with the Center for Disease Control (CDC), is conducting an epidemiological survey of hepatitis B in hemodialysis units. The objective of these studies is to determine to what degree hemodialysis patients and staff, and family contacts of these patients, can be classified as "high risk populations." These studies include identification of infecting source, method and prevalence of spread, identification of hepatitis B subtypes, and incubation time of the disease. Preliminary evidence indicates that hepatitis B is endemic in these populations, and data on the method of transmission are being collected.

The NIAID has awarded a contract for the establishment of a hepatitis study center in a population in which viral hepatitis is highly endemic. The study will include identification of infecting source, method and prevalence of spread, identification of hepatitis B subtypes, and incubation time of disease.

Passive Immunization: In FY 1973 the NIAID, in collaboration with the Veterans Administration (VA) hospitals, initiated studies to determine if high-titered HB Ab gamma globulin can prevent or modify hepatitis B infection in persons experiencing accidental parenteral exposure to this disease. Normal gamma globulin preparations confer some immunity to hepatitis A, but these preparations have little or no effect in preventing hepatitis B infection. Preliminary evidence from other studies suggests that gamma globulin containing HB Ab might be effective in preventing hepatitis B. In addition, the NIAID is collaborating with the VA hospitals on a second post-transfusion study, administering either conventional low titer or high titer anti-HB Ag gamma globulin to determine if these preparations lower the incidence of hepatitis B infections. This is a double-blind study so no results are currently available.

Immunohistochemical Assays: Currently one of the most sensitive techniques for the detection of antigen in tissue consists of immunohistochemical assay procedures. In FY 1973 the NIAID established a program for applying immunohistochemical assay techniques to the detection of hepatitis B antigens, particularly in primate specimens from animals involved in hepatitis infection studies.

ANTIVIRAL SUBSTANCES PROGRAM (ASP)

There are many viral diseases for which there is no prophylaxis or therapy presently available and which constitute serious public health problems. There is, therefore, a need for the development of antiviral substances for the treatment of these diseases. Planning for a program to help meet this need began in July 1969. At that time a thorough study of the field revealed that there was considerable increasing interest in this area and that interferon or interferon inducers were the most promising antivirals to exploit. This continues to be the case, although more recently there have been a number of case histories published which indicate that adenine

arabinoside (Ara-A) and iododeoxyuridine (IDU) have been effective against herpes encephalitis and other serious viral diseases. The program continues to be alert to new developments emanating from scientist-originated basic research so as to properly provide the needed stimulus to hasten the field by applying this knowledge to practical use in man. The general intent of the program is to serve as a focus for all activities on antivirals conducted by Extramural, Intramural and Collaborative Research of the National Institute of Allergy and Infectious Diseases and to some extent, other Institutes of the National Institutes of Health.

Following the advice of ad hoc committees and the Infectious Disease Committee, there has been a gradual shift of emphasis toward more clinical studies, as some of the original goals in basic studies to fill some of the gap areas are accomplished. There are also increased efforts in the area of antivirals other than interferon, as witness studies on Ara-A and IDU. The studies currently being pursued include the following.

Interferon Assay: In order to support and expedite research in the area of interferon, studies were undertaken to develop more rapid quantitative standardized assays. A variety of parameters involved in the assay systems were studied, and the information obtained did lead to considerable reduction in the time required for the performance of these assays. A rapid assay system utilizing the cytopathogenic effect (CPE) in plastic trays with several wells has been developed. There is one contract currently being supported to refine a new assay system which was developed by the contractor utilizing neuraminidase production as a quantitator of interferon. The neuraminidase assay does not require complete viral replication, which is an advantage. It can also be readily applied to a variety of interferon systems and thus has more universal appeal. It is expected that this contract will ultimately yield a simplified, rapid and sensitive assay which can be readily used in any laboratory doing interferon research.

Standards and Reagents: In collaboration with the Research Resources Branch, the ASP has successfully established reference reagents for rabbit interferon, mouse interferon, and interferon inducers [polyinosinic·polycytidylic acid (poly I·poly C)]. The two interferon preparations were originally prepared in the wet frozen state. The ASP has now made available lyophilized preparations of mouse interferon and also human interferon. These reagents are currently undergoing standardization analyses so that they can be assigned unitage which properly corresponds to the original materials established as the standards. A separate contract to produce highly specific antisera to mouse and human interferon is continuing. Antiserum to mouse interferon has been produced in rabbits and distributed to various workers in the field. Use of this reagent has helped to prove that there are two types of interferon produced in the mouse model. Use of this antiserum has also aided in developing purification methods for interferon using affinity chromatography. Techniques for developing antiserum to mouse interferon are being applied to developing antiserum to human interferon.

Purification: If exogenous interferon is to be used in man, there is a need for purification and concentration. The ultimate goal would be purification

to the extent that the chemical structure can be defined and eventually the interferon manufactured synthetically. Currently the use of affinity chromatography techniques for purifying interferon is being explored.

Production: If interferon proves effective in the treatment of human viral diseases, two methods of administration are available: the administration of exogenous interferon, produced in vitro, or endogenous interferon through the use of inducers. For the former there is a need for the development of a technique which will provide a more efficient, safe and economically feasible method of producing large quantities of human interferon. A wide variety of cells have been tested to determine their relative capacity to produce interferon using a series of inducers. There is very little difference between cells in their ability to produce interferon, but human amnion cells have some advantages. These comparative studies have been completed and the program is concentrating on superinduction studies. These studies have been very fruitful in that the yield from diploid cells has been increased 100-fold within the 2 1/2 years of study, and it is expected that this can be increased at least 10-fold more, which would put it in the realm of being economically feasible. A contract to provide the mechanism for large-scale production of interferon, once the ideal cell and induction system is established, is progressing well. In order to examine the potential clinical application of interferon inducers, studies are being undertaken to examine the relative merits of selected biological inducers.

Animal Models: In continued efforts to meet the urgent need of standardization in this field, several animal models have been established, including the mouse, rabbit, cat, ferret and pig. Data in these animal models are now being established using exogenous interferon and the inducer poly I·poly C. These data are then available for comparison with new antivirals as they develop. Currently these animal models are testing, in varying degrees of thoroughness, eleven candidate compounds which appear to have some clinical potential. If any one of these compounds proves to be efficacious and nontoxic, human studies can be considered.

Clinical Studies: The state of the art has progressed to the point where several clinical trials are now under way. The interferon inducer poly I·poly C has been standardized and is available to qualified investigators for clinical studies. It is currently being used in studies of herpetic keratoconjunctivitis and, to a much lesser extent, in some life-threatening diseases where no therapy is available.

Exogenous human leucocyte interferon is being used in prophylactic studies for the prevention of the recurrence of herpetic keratoconjunctivitis in patients who have a history of such a disease. It has been shown that individuals who have had two episodes of herpetic keratoconjunctivitis have a 40 percent chance of having a recurrence within the next two-year period. Exogenous interferon is also being studied in the treatment of disseminated herpes zoster, which is a major problem in immunologically compromised patients. These studies were selected for support because it is anticipated that interferon will have its most beneficial effect in disease states that are dependent on de novo viral replication for their pathogenesis.

Preliminary studies in serious viral infections such as herpes encephalitis have shown that IDU and Ara-A have definite beneficial results. A study has therefore been developed to test, in a comparative fashion, IDU, Ara-A and placebo to determine whether both or either of these compounds does indeed have clinical efficacy. Because Ara-A has been shown to be non-toxic in man, it is also being studied for a beneficial effect against disseminated herpes zoster and herpes virus hominis in the immunologically compromised patient where either of these viruses presents serious problems. It is expected that Ara-A will also eventually be shown to be beneficial against neonatal herpes and cytomegalovirus infections, which continue to be problems with the newborn.

The research sponsored by this program continues in its effort to attempt to bridge the gap from scientist-originated research to practical use of this information in man.

UNIVERSITY OF ALABAMA - (NIH-72-2532)

Title: Operation of a Single Facility to Study Treatment of Serious Viral Infections with Chemotherapeutics

Contractor's Project Director: Charles A. Alford, Jr., M.D.

Project Officers: George J. Galasso, Ph.D.; Michael Worthington, M.D.

Objectives: Studies in animals and preliminary studies in man indicate that 9-B-D Arabinofuranosyl adenine (Ara-A) and iododeoxyuridine (IDU) are effective antiviral compounds. This contract will serve to demonstrate whether these compounds are indeed useful in the clinical treatment of viral diseases of man.

Major Findings: The first year of this contract was spent in refining the clinical protocols to test Ara-A and IDU against herpes encephalitis and Ara-A against disseminated herpes zoster and herpes virus hominis in immunosuppressed patients. Simultaneously drug and placebo were obtained from the two manufacturers. In order to obtain sufficient numbers of patients for the study to proceed in an efficient and expeditious manner, eight other participating clinics were sought and obtained to follow the protocols as subcontractors of this primary contractor. All this has been accomplished and cleared with the various reviewing committees such as the Clinical Review Committee and the Food and Drug Administration. The study is currently underway with patients being entered into the study at the expected rate.

Significance to NIAID Programs and Biomedical Research: The purpose of the Antiviral Substances Program of the NIAID is to determine which of the newly developed antivirals have clinical potential and then to assist in bringing these drugs as expeditiously as possible to practical application. The drugs under study seem to have this potential and hopefully can be shown to be clinically useful within the next few years.

Proposed Course of Project: It is anticipated that this project will be continued through June 1976 under option to renew, dependent on productivity and continued need.

Date Contract Initiated: June 26, 1972

Current Annual Level: $250,000

THE AMERICAN RED CROSS (NIH 72-2522)

Title: Hepatitis Testing Laboratory

Contractor's Project Director: G. A. Jamieson, Ph. D.

Project Officer: June K. Dunnick, Ph. D.

Objectives: The contractor shall establish a Hepatitis Testing Laboratory to do tests for hepatitis B antigens and antibodies. In addition, the testing laboratory is involved in developing better methods to test for hepatitis B antigens and antibodies.

Major Findings: The American Red Cross has established a Hepatitis Testing Laboratory capable of testing up to 7500 samples per month for hepatitis B antigen and antibody. Tests offered by the testing laboratory include radio-immunoassay tests for antigen and antibody, passive hemagglutination techniques and subtype identification.

Significance to NIAID Program and Bio-Medical Research: The testing laboratory provides laboratory support for other ongoing NIAID programs including animal model research, passive immunization studies and epidemiology studies. This laboratory also offers its services to other NIH hepatitis research projects. This laboratory is part of the NIAID program to find more sensitive assay procedures for hepatitis B antigens and antibodies.

Proposed Course of Project: The testing laboratory will probably function for at least 3 more years.

Date Contract Initiated: June 22, 1972

Current Annual Level: $560,000 (estimate)

BAYLOR UNIVERSITY (NIH 73-2506)

Title: Development and Evaluation of Methods for Control of Viral and Mycoplasmal Respiratory Diseases

Contractor's Project Director: Robert B. Couch, M.D.

Project Officers: Herbert Reynolds, M.D. and George Galasso, Ph. D.

Objectives: To test experimental respiratory virus vaccines and chemoprophylactic agents, and to develop and evaluate principles for development and the evaluation of such agents.

Major Findings: Inactivated M. pneumoniae vaccines have been successfully evaluated by this contractor during the past year. Currently he is testing M. pneumoniae challenge pools by the intranasal drop and aerosol routes. An aerosol challenge dose that results in atypical pneumonia is being determined. This challenge will be used to determine if the new, very potent, lots of inactivated M. pneumoniae vaccines are protective.

Inactivated subunit adenovirus vaccine type 5 is being evaluated in children about 12 years of age. Studies will be initiated step-wise into younger children if the vaccine appears safe and immunogenic in this age group.

Surveillance on recipients of inactivated influenza virus vaccines has been maintained. An epidemic occurred in January 1973. Data on protective levels of heterotypic antibody is being gathered and evaluated.

Significance to NIAID Programs and Bio-Medical Research: This group possesses the experience and facilities to carry out both open and closed clinical studies including challenge studies which are necessary for the evaluation of various vaccines in the program.

Proposed Course of Project: This contract will probably be in effect until December 19, 1977. Incremental funding is planned.

Date Contract Initiated: December 20, 1972

Current Annual Level: $210,000

BUREAU OF BIOLOGICS, FDA (Interagency Agreement)

Title: Support of chimpanzee colony at Holloman Air Force Base

Contractor's Project Director: Dr. Lewellys Barker, FDA
Dr. F. Coulston, Albany Medical College

Project Officer: Dr. June K. Dunnick

Objectives: Provide boarding and maintenance for a breeding colony of at least forty female chimpanzees who are expected to breed annually. The sero-negative offspring will be available for use in hepatitis studies and other studies as designated by the project officer.

Major Findings: None

Significance to NIAID Program and Bio-Medical Research: Animal model systems for hepatitis B are necessary for vaccine efficacy and potency tests. The chimpanzee has been demonstrated to be an excellent animal model for these studies. This contract will provide chimpanzees to do the necessary hepatitis vaccine evaluation studies. The initial studies will consist of titering known infectious hepatitis B material in the chimp. Following these studies inactivated vaccine preparations (such as boiled materials) will be tested. This contract is funded jointly by the CDC, FDA and NIAID.

Proposed Course of Project: It is estimated that this facility will provide animals to the Hepatitis Program for at least 3 years.

Date Contract Initiated: June 1973

Current Annual Level: $50,000

UNIVERSITY OF CALIFORNIA (NIH 72-2520)

Title: Therapeutic and Prophylactic Effects of Interferon Inducers on Herpetic and Adenoviral Infections of the Human Eye

Contractor's Project Director: Chandler R. Dawson, M.D.

Project Officers: Samuel Baron, M.D. and George J. Galasso, Ph. D.

Objectives: This is a study to evaluate the value of polyinosinic·polycytidylic acid, an inducer of interferon, in the treatment of viral diseases of the human eye, primarily herpetic keratoconjunctivitis.

Major Findings: Although this contract was begun in June 1972, no clinical trials could be initiated prior to March 26, 1973, when final packaging and safety testing of the drugs was completed under a purchase order contract to the NIH. During this time period, 46 patients were seen with possible herpetic lesions and 26 with possible other viral infections.

Significance to NIAID Programs and Bio-Medical Research: Polyinosinic·polycytidylic acid is an effective inducer of interferon. This topical treatment, if effective, would solve an important medical disease problem which is common and very painful to the patient. The current therapy, although useful, needs improvement.

Proposed Course of Project: To terminate June 24, 1974

Date Contract Initiated: June 25, 1972

Current Annual Level: $35,866 (Estimate)

UNIVERSITY OF CALIFORNIA - SAN DIEGO (NIH 70-2263)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: James D. Connor, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: A collaborative study to compare reactivity and immunogenicity of four smallpox vaccines (calf lymph, CAM, CV-1, and Elstree strains of vaccinia) administered at $10^6$, $10^7$, and $10^8$ pkfu percutaneously and $10^3$, $10^4$, and $10^5$ pkfu subcutaneously is in progress. These vaccinations were all followed after approximately six months by percutaneous vaccination with calf lymph vaccine at $10^8$ pkfu. Although very little of the post-challenge antibody data is available, the CV-1 vaccine appears to be less effective in inducing antibody formation than the other three vaccines. It appears that fewer complications are associated with subcutaneous vaccination and that fewer complications follow standard percutaneous inoculation in individuals previously given subcutaneous vaccine, although the same percentage of complications are seen in those who were previously given percutaneous vaccine.

Significance to NIAID Programs & Bio-Medical Research: At the present time routine smallpox immunization are not being given in the United States. Outbreaks of smallpox continue to occur in other countries. It is hoped that the present collaborative study will result in information that can be used to determine a better immunization procedure - for use whenever persons need to be vaccinated.

Proposed Course of Project: Contract to terminate June 21, 1973

Date Contract Initiated: June 22, 1970

Current Annual Level: $24,247 (6 months)

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO (NIH 72-2500)

Title: Evaluation of the antigenicity of pneumococcal polysaccharide vaccines in children

Contractor's Project Director: Arthur J. Ammann, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: This study seeks to evaluate 14 type specific monovalent pneumococcal polysaccharide vaccines in children of different age groups. It is the purpose of this study to determine the degree and duration of antibody response for each monovalent preparation. After reactivity and antigenicity have been established for each monovalent preparation, these studies will be extended to the evaluation of a polyvalent vaccine in children of the appropriate age groups.

Major Findings: The major thrust of the project has resulted in 150 children being immunized with 14 monovalent vaccines and pilot studies with a candidate hexavalent vaccine in approximately 21 young children, ranging in age from 6 months to 13 years. There were no serious adverse local or systemic reactions associated with any of the prototype vaccines undergoing clinical evaluation.

To date, the serological response of recipients of monovalent vaccines (types 1, 3, 8, 14 and 18) have been measured by a passive HA test developed by the contractor. These studies were designed to assess antigenicity in children to a standard 50 ug dose of each polysaccharide administered by the subcutaneous route. Two weeks following immunization, there was a 4-fold or greater increase of HA antibody to these five serotypes of polysaccharide.

Significance to NIAID Program and Bio-Medical Research: Studies of Dr. Virgil Howie and others have clearly shown that Streptococcus pneumoniae is a major etiologic agent of acute otitis media in infants and young children. There is preliminary evidence to show that children suffering with sickle cell anemia are at high risk to pneumococcal infection. Frequently many of these cases culminate in a severe form of meningitis. It is imperative to make an assessment of the response of young children to pneumococcal polysaccharides. If the antibody response is favorable, it is conceivable that a pediatric formulation of a polyvalent vaccine could be directed to reduce the excessive morbidity of these commonly occurring acute infectious illnesses.

Proposed Course of Project: This contract will be reviewed and evaluated prior to April 30, 1974 and decision made on renewal depending on program requirement.

Date Contract Initiated: May 1, 1972

Current Annual Level: $52,000

CENTER FOR DISEASE CONTROL, ATLANTA, GEORGIA (Interagency Agreement)

Title: Study of hemodialysis-associated hepatitis in two dialysis units

Contractor's Project Director: John A. Bryan, M.D.

Project Officers: Robert H. Purcell, M.D. and June K. Dunnick, Ph. D.

Objectives: To conduct a hepatitis epidemiological survey of two hemodialysis units to 1) establish the incidence of hepatitis B antigen, hepatitis B antibody and clinical viral hepatitis in the hemodialysis setting, and 2) identify the factors involved in transmission of hepatitis B or HB Ag positivity in the hemodialysis setting, and 3) determine the risk of acquiring hepatitis B or HB Ag to household contacts of dialysis patients and staff.

Major Findings: The CDC has negotiated two subcontracts with Emory University and the University of Mississippi to study hemodialysis-associated hepatitis in renal dialysis units located at these universities. The studies were begun in March 1973, so at this time, there are no major findings to report.

Significance to NIAID Program and Bio-Medical Research: This project is part of the NIAID's Hepatitis Program to determine what are the "high risk" populations for hepatitis B, and to study what would be the best means of preventing hepatitis B in these populations.

Proposed Course of Project: The project will be in progress for almost two more years.

Date Contract Initiated: July 1, 1972

Current Annual Level: $63,038

NATIONAL CENTER FOR DISEASE CONTROL (Intraagency Agreement) 71A17

Title: Smallpox Serologies

Contractor's Project Director: James Nakano, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: Perform vaccinia hemagglutination-inhibition (HI) and serum neutralization tests (SN) on 1200 serums.

Major Findings: Contractor is acting as the reference laboratory for the four collaborative smallpox clinical studies. The reference laboratory is responsible for testing 1/4 of the serum samples and correlating results from the other laboratories. Results obtained by the reference laboratory indicate that the four clinical investigators are obtaining similar results with the HI test. The SN tests are not correlating as well and some of them will be repeated.

Significance to NIAID Programs & Bio-Medical Research: At the present time routine smallpox immunization are not being given in the United States. Outbreaks of smallpox continue to occur in other countries. It is hoped that the present collaborative study will result in information that can be used to determine a better immunization procedure - for use whenever persons need to be vaccinated.

Proposed Course of Project: To continue through June 30, 1973.

Date Contract Initiated: July 1, 1970

Current Annual Level: $21,600

UNIVERSITY OF CHICAGO (PH 43-68-83)

Title: Immunization of humans with group A streptococcal proteins

Contractor's Project Director: Albert Dorfman, M.D., Ph. D.

Project Officers: Roger Cole, M.D. and Richard E. Horton, M.D.

Objectives: The purpose of these studies includes clinical trials to demonstrate the following values of antigenicity in adults immunized with streptococcal M-protein vaccines: 1. levels of antibody, including bactericidal, at intervals of one year or longer; 2) protection against homologous challenge at 6 months or longer after immunization; and 3) effect of booster doses on antibody levels and protection against homologous challenge. In addition, challenge studies will be expanded to adult volunteers immunized with trivalent combinations of serotypes of 3, 6 and 12 M-protein vaccines.

Major Findings: Studies completed by the contractor during the last year include immunization of 19 adults by subcutaneous injection and 16 by intranasal application. These 35 vaccinees were challenged with live type 1 streptococci along with 40 control subjects. In addition, 30 children have been immunized by the intranasal route. All vaccinees were studied for serum antibody responses by 3 methods: passive hemagglutination assay, complement fixation and bactericidal assay. Response of subjects challenged with live streptococci were measured by a graduated point system. Criteria for evaluating streptococcal upper respiratory tract infection included temperature and WBC response; results of throat cultures, and a number of signs and symptoms including pharyngeal and tonsillar erythema, enlarged cervical lymph nodes, tonsillar exudate, sore throat, headache and malaise. All infectivity studies were limited to adults who were selected after screening for delayed hypersensitivity to M-protein and hypersensitivity to penicillin. Although the degree of immunity produced is not sharply defined, the recorded data suggest a significant level of protection by both methods of vaccination (parenteral or intranasal).

Significance to NIAID Program and Bio-Medical Research: Streptococcal disease and its sequelae, namely rheumatic fever and glomerulonephritis are significant health problems. An effective safe vaccine that reduces the prevalence of these illnesses would be of great importance.

Proposed Course of Project: This contract will be reviewed and evaluated prior to May 22, 1974, and decision made on renewal depending on program requirements.

Date Contract Initiated: January, 1968

Current Annual Level: $73,697

CHILDREN'S HOSPITAL MEDICAL CENTER, HARVARD MEDICAL SCHOOL (NIH 71-2196)

Title: Studies on *Hemophilus influenzae* type b vaccine

Contractor's Project Director: David Smith, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1.) Provide services of a reference laboratory for the microbiological identification and serotyping of *Hemophilus influenzae* isolated from clinical cases of meningitis; 2.) apply a radioimmunoassay to measure antibody response to *H. influenzae* type b infection and active immunization with polyribose phosphate (PRP) vaccine; 3.) conduct vaccine testing in infants with PRP antigen to determine safety and dose response data.

Major Findings: During the past contract period, 184 isolates of *H. influenzae* from healthy children and 65 from children with systemic disease were serotyped; all but one of these isolates were type b. Serum antibody and antigen titers were performed on 77 children with systemic *H. influenzae* disease. Antibody activity during hospitalization was considerably lower than that of healthy age-matched children; rises in anti-PRP titers during convalescence correlated with age, with reduced responses among younger children, and antigenemia. The results of radioimmunoassay (RIA) have been compared to those obtained with bactericidal (BC) and passive hemagglutination (PHA) methods. Four hundred fifty ng/ml anti-PRP killed all tested *H. influenzae* type b in the standardized bactericidal assay. Anti-PRP activities of 159 adults, 63 newborns and 5 agammaglobulinemic children receiving prophylactic gamma globulin revealed that each of these presumably immune groups had median antibody titers of less than 400 ng/ml. Activities in 100 children under one year of age were obtained and appeared to mirror the age-related susceptibility to *H. influenzae* type b illnesses. Two new lots of PRP vaccine have been prepared; one lot is the original purified antigen packaged at a lower concentration level (20 ug/ml) and the other is a less purified preparation which is being studied for toxicity and immunogenicity in animal models.

Significance to NIAID Program and Bio-Medical Research: Improved methods are needed for microbiological and serological diagnosis of *H. influenzae* type b infection of children. A highly sensitive procedure such as the RIA is required to measure antibody response to natural disease prototype vaccines used in immunization studies. An effective vaccine is needed to give high levels of protection against *H. influenzae* infections.

Proposed Course of Project: To be reviewed and evaluated prior to end of current contract year.

Date Contract Initiated: June, 1971

Current Annual Level: $111,286 (estimated)

CHILDREN'S HOSPITAL RESEARCH FOUNDATION, COLUMBUS, OHIO (NIH 69-2062)

Title: Rhinovirus Reference Center

Contractor's Project Director: Vincent V. Hamparian, Ph. D.

Project Officers: Albert Kapikian, M.D. and Martha Mattheis

Objectives: To compare by serological methods suitable candidate prototype rhinoviruses received from collaborating laboratories throughout the world in order to define the number of prevalent serotypes and to allow an agreement to be reached by interested workers on a final numbering system.

Major Findings: A total of 89 rhinoviruses were defined as prime strains at the close of phase II of the rhinovirus typing program. Twenty to 25 new rhinovirus strains will be defined in phase III. More than 100 untyped viruses that are not part of phase III have been submitted to the rhinovirus reference center. Undoubtedly many of these will be the same as some of the phase III candidates.

This program will be terminated this year with about 110 rhinovirus serotypes having been defined. The contract has achieved its objectives because most of the rhinoviruses have been typed and numbered. It is recognized that a few additional rhinoviruses will be found in the coming years.

Significance to NIAID Program and Bio-Medical Research: Rhinoviruses are the major cause of the "common cold". In order to find prophylactic methods for combating the "common cold" it has been necessary to know the causative agents. It is unlikely that a vaccine will be effective because of the large number of types, the lack of prevalent types nation-wide, and the low amount of cross-reactivity among most types.

Proposed Course of Project: This contract will terminate June 30, 1973.

Date Contract Initiated: May 1, 1969

Current Annual Level: $12,041 (3 months)

RESEARCH FOUNDATION OF CHILDREN'S HOSPITAL, WASHINGTON, D.C. (NIH 71-2091)

Title: Evaluation of Respiratory Virus Vaccines in Infants and Children

Contractor's Project Director: Robert Parrott, M.D.

Project Officers: Robert Chanock, M.D. and Martha Mattheis

Objectives: 1) To study presumably attenuated viruses as potential vaccines. 2) To study factors in the resistance to and pathogenesis of illness resulting from natural infection with respiratory viruses, particularly RS and parainfluenza viruses. 3) To study the "paradoxical" effect following intramuscular injection of inactivated RS vaccine.

Major Findings: Currently this contractor is evaluating the live influenza $A_2$ ts 1 (E) virus vaccine in children at least 4 years old, for safety and antigenicity. The vaccine has infected all 10 of the children who have been immunized to date. Very mild rhinitis occurred in three of the children.

Respiratory syncytial virus vaccine (ts-1 mutant) has been administered intranasally to 4 children by drops. Vaccinees receiving the $10^{-1}$ dilution shed virus while vaccinees receiving the $10^{-2}$ dilution did not. No illness occurred. Serological data have not as yet been obtained. It is possible that the vaccinees receiving the $10^{-2}$ dilution will have rises in antibody titer to RSV indicating that they were infected.

Significance to NIAID Program and Bio-Medical Research: Respiratory syncytial virus and the parainfluenza viruses are the major known causes of severe respiratory illness in young children. Influenza A has been shown to be an important cause of lower respiratory tract illness during epidemics.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal under the option to renew clause at the end of the contract year (December 1973).

Date Contract Initiated: December 7, 1971. This contract was previously number PH43-62-473.

Current Annual Level: $133,325

UNIVERSITY OF COLORADO (NIH 70-2264)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: C. Henry Kempe, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: A collaborative study to compare reactivity and immunogenicity of four smallpox vaccines (calf lymph, CAM, CV-1, and Elstree strains of vaccinia) administered at $10^6$, $10^7$, and $10^8$ pkfu percutaneously and $10^3$, $10^4$, and $10^5$ pkfu subcutaneously is in progress. These vaccinations were all followed after approximately six months by percutaneous vaccination with calf lymph vaccine at $10^8$ pkfu. Although very little of the post-challenge antibody data is available, the CV-1 vaccine appears to be less effective in inducing antibody formation than the other three vaccines. It appears that fewer complications are associated with subcutaneous vaccination and that fewer complications follow standard percutaneous inoculation in individuals previously given subcutaneous vaccine, although the same percentage of complications are seen in those who were previously given percutaneous vaccine.

Significance to NIAID Programs & Bio-Medical Research: At the present time routine smallpox immunization are not being given in the United States. Outbreaks of smallpox continue to occur in other countries. It is hoped that the present collaborative study will result in information that can be used to determine a better immunization procedure - for use whenever persons need to be vaccinated.

Proposed Course of Project: Contract terminated on March 12, 1973

Date Contract Initiated: June 15, 1970

Current Annual Level: $8,575

UNIVERSITY OF COLORADO - (NIH-72-2501)

Title: Respiratory Viral and Bacterial Polysaccharide Vaccines

Contractor's Project Director: Kenneth McIntosh, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: Clinical evaluation of live virus vaccines (respiratory syncytial virus, parainfluenza viruses), and inactivated adenoviruses), with major emphasis on the evaluation of live respiratory syncytial virus vaccine in closed populations of infants to ascertain infectivity, lack of virulence and production of antibody; in semi-closed or carefully monitored open populations of children and infants to obtain additional information of infectivity, lack of virulence, production of antibody and transmissibility; and in semi-closed, carefully monitored populations of children with asthma, to obtain information on infectivity, degree of virulence, production of antibody and transmissibility.

With modified dosages continue clinical studies to evaluate reactivity and antigenicity of candidate bacterial polysaccharide vaccines, with a major emphasis on Hemophilus influenza type b polyribose phosphate vaccine (PRP), as well as meningococcal groups A and C vaccines in infants.

Major Findings: The principal investigator has made significant contributions in the evaluation of respiratory vaccines since the original contract was awarded in 1962. Major findings since 1970 are described as follows:

1. Studies with live RSV-A2-26$^{o}$ adapted vaccine in asthmatic children indicated that the vaccine was well tolerated. Children (13/13) with serum neutralizing antibody levels of 1:800 or less had vaccine virus "takes," whereas four of five children with antibody levels greater than 1:1600 were not infected with vaccine virus. A natural RSV outbreak occurred in November and December, 1970. Nasal wash antibody studies indicated that protection was afforded to 8/8 vaccinated children, while 5/10 from the placebo group were infected during this outbreak. There was no evidence to suggest that natural RSV illness after live vaccine was worse than natural RSV illness after placebo administration. This vaccine virus was shown to be protective if an epidemic occurred 4 to 5 months following its administration, but the long term protective value of this vaccine would require further evaluation.

2. Studies with the RSV-ts-1 mutant vaccine in asthmatic children have demonstrated that it was infective in younger children and did not cause excess wheezing. Undiluted vaccine was associated with URI symptoms in younger children; however, 3/7 had concomitant infections with Group A streptococci and coronavirus OC43 so the cause of the URI symptoms is not clear. There was a tendency for the virus to spread to contacts and, in some instances, to revert in vivo and regain its ability to grow at 37$^{o}$ and 38$^{o}$C. Preliminary studies with reduced dosage ($10^{2.9}$ and $10^{1.9}$ $TCID_{50}$) have been initiated. In the first trial, 4/6 vaccinees shed virus. URI symptoms were rare. One child had mild unilateral otitis media on day 12 following

mild rhinorrhea beginning on day 9. This disease may have been due to vaccine virus, but studies of concomitant infections have not been completed.

3. A longitudinal study of immunogenicity of group A and C meningococcal polysaccharide and H. influenzae PRP vaccines was carried out in 58 children (4-12 years of age) at the Colorado State Home and Training School, and observations made for a period of 18 months. In a second trial study, 36 normal children, aged 1-5, were randomly immunized with group C meningococcal vaccine at two dose levels (20 and 50 ug). A later trial included 29 children who received PRP in one arm and meningococcal A and C vaccine in the other arm. The immunizing dose was 25 ug of each vaccine. Serum samples were collected at 0 and 21-28 days after immunization. Serological response of recipients of PRP vaccine has not been completed. Initial paired sera are being analyzed by the radioimmunoassay of Dr. Porter Anderson at the Children's Medical Center, Boston. The antibody response to meningococcal vaccine was measured by the indirect hemagglutination (IHA) and bactericidal (BC) tests. There was an IHA antibody rise within two weeks following immunization with 50 ug of group A or group C meningococcal polysaccharides. Vaccinees receiving group A antigen raised IHA antibody titers in the range of 1:64 by two weeks. These titers leveled off at 1:8 by six months postimmunization (PI).

A limited study with sera from 11 recipients of group C vaccine were assayed by the bactericidal test and compared with IHA antibody levels. In contrast to the gradual fall of IHA antibody, the bactericidal antibody level remained elevated during the 16 month period of observation. It would appear from this preliminary information that vaccinees who develop an initial group C bactericidal antibody of 1:64 or greater tended to maintain titers of 1:16 or higher when analyzed at the 16 month interval.

Significance to NIAID Programs and Bio-Medical Research: Asthmatic children contracting RSV infection experience a high incidence of acute wheezing attacks and pronounced pulmonary changes on X-ray examination. These studies will establish the role of an attenuated RS vaccine to induce immunity in this group of allergic children.

Studies concerned with the administration of H. influenzae type b, meningococcal (group A and C) to children are directed to developing effective immunization programs for H. influenzae and meningococcal meningitis of young children and infants.

Proposed Course of Project: This contract will be reviewed and evaluated before the next anniversary date, June 1974.

Date Contract Initiated: April 4, 1972

Current Annual Level: $84,780

UNIVERSITY OF CONNECTICUT (NIH 72-2502)

Title: Clinical evaluation of bacterial polysaccharide vaccines

Contractor's Project Director: Martha Lepow, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1. Complete phase I clinical evaluation and analysis of the serological response of infants to immunization with meningococcal groups A and C polysaccharide vaccines. Specifically, this includes determining the antibody response of infants to two dose levels (25 ug and 100 ug) of group A and group C vaccines administered at different ages (3 months and 7 months). 2. Assess the individual antibody response of 3 month old infants immunized simultaneously with optimal dosages of meningococcal groups A and C vaccines and H. influenzae type b (PRP). 3. Sera from each vaccinee will be obtained at 0, 3, 7, 12 and 13 months following immunization and submitted to the Infectious Disease Branch serological testing laboratories for quantitative antibody determinations.

Major Findings: This contractor has made excellent progress during the first contract year. One hundred seventy-six infants, approximately 3 months old, were immunized with 62.5 or 100 ug of group A or with 25 or 100 ug of group C meningococcal vaccine. No major systemic reactions occurred, although 14/159 or 9% of the infants were classified as being irritable by their mothers for 12-24 hours following vaccination. No significant local or systemic reactions have been observed following vaccine booster doses given at 7 months of age.

Preliminary serological results with the radio-antigen binding (RAB) procedure of Dr. Gotschlich, 5 weeks post-immunization, indicate no significant difference in antibody response between 25 and 100 ug of group C vaccine. In limited serological studies completed to date, the evidence suggests children 18 months and older are able to raise larger levels of group specific antibody, compared to infants less than 12 months of age. Further dose-response studies by the contractor will focus on infants under the age of six months.

Significance to NIAID Program and Bio-Medical Research: The peak incidence of bacterial meningitis in children is between 6 and 24 months of age. Therefore, it is essential to determine the optimal dose, frequency and time of immunization in order to be able to protect this most susceptible age group by immunization.

Proposed Course of Project: This contract will be reviewed and evaluated prior to April 1974, and decision made on renewal depending on program requirements.

Date Contract Initiated: April 1972

Current Annual Level: $50,891

CORNELL UNIVERSITY MEDICAL COLLEGE (NIH 70-2065)

Title: Study of the antigenicity and immunogenicity of Mycoplasma pneumoniae vaccines.

Contractor's Project Director: Laurence Senterfit, Sc. D.

Project Officers: Robert Purcell, M.D. and Martha Mattheis

Objectives: 1) Collaborate with Drs. Somerson (Ohio State University) and Hamparian (Children's Hospital, Columbus, Ohio) in the production of inactivated M. pneumoniae vaccine by performing the required animal studies, 2) study the immunogenicity and antigenic structure of temperature sensitive (ts) mutants of M. pneumoniae, 3) complete the development of methods for the qualitative and quantitative measurement of antibody to specific determinants of the Mycoplasma membrane, 4) conduct serological testing of sera from clinical trials with HRC and Ohio State vaccines, 5) to study the adjuvant effect of polynucleotides on viral, mycoplasmal and bacterial vaccines.

Major Findings: An accelerated stability test of the potency of inactivated M. pneumoniae vaccines is in progress. The vaccine appears stable for 1 month when stored at -70°C, -20°C, and +4°C, but not at +37°C. The vaccines will be stored at these temperatures for one year and will be tested for antigenicity (stability) at intervals during that time period.

Two large lots of inactivated M. pneumoniae vaccine have been prepared at Ohio State University under a separate contract. The animal tests for satety and potency of these lots of vaccine were performed at Cornell University. The vaccines contained two times as much protein as previous lots and were ten times more antigenic in hamsters and guinea pigs. These lots of vaccine are being clinically evaluated by investigators at different institutions, and the Cornell University contract is serving as a serological reference laboratory for these studies.

Significance to NIAID Programs and Bio-Medical Research: There is a need for a Mycoplasma pneumoniae vaccine in young adults, especially in recruit camps. This contract will perform the majority of the serological assays from any field trials and a portion of the assays from all other trials.

Proposed Course of Project: The contract will be reviewed and evaluated prior to the anniversary date, January, 1974.

Date Contract Initiated: January 18, 1970

Current Annual Level: $37,882

ELI LILLY AND COMPANY (PH43-68-1280)

Title: Production of specific immunogenic polysaccharide vaccines from selected serological types of streptococcus pneumoniae

Contractor's Project Director: Robert S. Baker, Ph. D.

Project Officer: Richard E. Horton, M.D.

Objectives: Complete production of at least 25 gms of each of pneumococcal polysaccharide types (1-9, 12, 14, 18, 19 and 23) and formulate prototype polyvalent vaccines.

Major Findings: The currently available polysaccharides have been shown to be safe and essentially non-reactive when administered in monovalent form. Final packaging and testing for sterility, identity and general safety has been accomplished for one hexavalent vaccine with serotypes (1, 3, 4, 7, 8 and 12), a second hexavalent vaccine with serotypes (2, 5, 6, 14, 18 and 19), a dodecavalent vaccine with serotypes (1-8, 12, 14, 18 and 19), and an octovalent vaccine with serotypes (1, 3, 6, 7, 14, 18, 19 and 23). Preliminary clinical trials are in progress to demonstrate these vaccines are safe and immunogenic in human volunteers.

Significance to NIAID Programs and Bio-Medical Research: Vaccines forthcoming from this contract represent a highly significant segment of activity of the overall pneumococcal vaccine program to determine the efficacy of a polyvalent vaccine to reduce the rising incidence of pneumococcal disease.

Proposed Course of Project: It is anticipated that the contractor will complete the assigned work task by June, 1973.

Date Contract Initiated: June, 1968

Current Annual Level: 0

FLOW LABORATORIES (PH-43-NIAID-68-1309)

Title: Operation of Virus and Tissue Culture Laboratories

Contractor's Project Director: Raymond V. Gilden, Ph. D.

Project Officers: Robert M. Chanock, M.D. and Martha J. Mattheis

Objectives: 1) To perform research and development activities in support of the Laboratory of Infectious Diseases (LID), NIAID respiratory virus and mycoplasma research program; 2) to develop and prepare tissue cultures for LID's program and for the program described in 1.

Major Findings: During the past year a 4 liter lot of live influenza virus, type A HK ts-1 "E" recombinant vaccine, grown in the allantoic cavity of RIF free embryonated eggs was produced, safety tested and packaged. A 5-8 liter lot of London 1972 influenza virus type A (H3N2) ts-1 "E" recombinant vaccine is in the process of being prepared in embryonated eggs.

Six different virus pools totaling 5.4 titers of allantoic fluid were produced for LID personnel. Four experimental lots of virus vaccines produced at LID are being safety tested.

Dr. Potash is collaborating with Dr. Gerin in studies on the preparation of stable inactivated parainfluenza virus vaccines. The first lot of parainfluenza virus type 3 has been shown to be antigenic and stable in accelerated stability assays in hamsters.

Significance to NIAID Programs and Bio-Medical Research: This contract provides unique tissue cultures essential to various research programs of the LID. It also provides vaccine production and/or safety testing of vaccines produced by LID or by other IDB contractors. In addition, genetic mutants suitable for the preparation of parainfluenza viruses and respiratory syncytial virus vaccines are being developed under Dr. Chanock's direction.

Proposed Course of Project: Contract will be reviewed and evaluated at the end of the current contract period.

Date Contract Initiated: June 23, 1968

Current Annual Level: $436,106

INDIAN HEALTH SERVICE, HEALTH SERVICES AND MENTAL HEALTH ADMINISTRATION
(Interagency Agreement #70-A-11)

Contractor's Project Director: George E. Bock, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: To conduct clinical and laboratory surveillance among Navajo Indians living in the Gallup Service Area and establish an incidence rate for pneumococcal pneumonia, otitis media and meningitis in preparation for a pneumococcal polysaccharide vaccine field trial.

Major Findings: This collaborative epidemiological study between the Navajo Indian Health Area and the University of New Mexico continues to provide data on the incidence rates for pneumococcal pneumonia, acute otitis and meningitis among the Navajo Indians receiving medical health care at the Gallup PHS Indian Hospital. Analysis of the data gathered between July 1, 1972 and March 31, 1973 revealed a total of 258 cases of pneumonia (156 cases of in-patient pneumonia and 102 cases of out-patient pneumonia). A crude incidence rate for pneumococcal pneumonia in individuals 15 years or older is estimated at 13 cases per 1000 individuals per year. Pneumococcal isolates were recovered from 74% of the total cases of pneumonia. Fifty-seven percent of the pneumococcal isolates consisted of serotypes (1-8, 12, 14, 18 and 19) that would be formulated in a polyvalent vaccine. Studies during FY 1973 add further support to the exceedingly high incidence of pneumococcal disease and the suitability of the population for a double-blind vaccine trial to demonstrate efficacy.

Significance to NIAID Program and Bio-Medical Research: To demonstrate efficacy of prototype pneumococcal polyvalent vaccines, populations with significantly high incidence rates of pneumococcal disease are required. This particular population fulfills this basic requirement and would stand to benefit immensely from an immunoprophylactic agent of this nature.

Proposed Course of Project: This interagency agreement will be reviewed and evaluated for renewal at the end of the 1973 fiscal year.

Date Agreement Initiated: July, 1969

Current Annual Level: $66,981

UNIVERSITY OF IOWA (NIH 72-2519)

Title: The Effects of Interferon Inducers on Infections of the Eye

Contractor's Project Director: Bruce Golden, M.D.

Project Officer: Samuel Baron, M.D., George J. Galasso, Ph. D.

Objectives: This is a study to evaluate the value of polyinosinic · polycytidylic acid, an inducer of interferon, in the treatment of viral diseases of the human eye, primarily herpetic keratoconjunctivitis.

Major Findings: Although this contract was begun in June, 1972, no clinical trials could be initiated prior to March, 1973 when final packaging and safety testing of the drugs was completed under a purchase order contract to the NIH. The contract will terminate in June when the principle investigator moves to another location. Some patients with acute herpetic keratoconjunctivitis and some with adeno keratoconjunctivitis will be treated between March and June, 1973.

Significance to NIAID Programs and Bio-Medical Research: Polyinosinic · polycytidylic acid is an effective inducer of interferon. This topical treatment, if effective, would solve an important medical disease problem which is common and very painful to the patient. The current therapy, although useful, needs improvement.

Proposed Course of Project: To terminate June 25, 1973

Date Contract Initiated: June 26, 1972

Current Annual Level: $33,880 (Prior year support)

KAISER PERMANENTE MEDICAL GROUP OF SAN FRANCISCO - (NIH-72-2513)

Title: Epidemiological study of pneumococcal disease

Contractor's Project Director: Marvin A. Freid, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) Based on appropriate clinical examination, including chest roentgenograms, determine a firm incidence rate for pneumococcal pneumonia in subscribers in respect to age, sex, pre-existing disease and place of residence. 2) Determine the prevalence of pneumococcal serotypes responsible for pneumococcal disease by appropriate bacteriological examination of blood sputum, naso-pharyngeal secretions or other body fluid specimens.

Major Findings: This contract for the collection of epidemiologic data on pneumococcal pneumonia illness in 125,000 subscribers in the Kaiser Permanente health plan was awarded March 1, 1972. A surveillance program supported by a separate bacteriology research laboratory became operational by May 1, 1972. Interim data on the prevalence of disease is now available for a 6 1/2 to 7 month period from May 1 to November 15, 1972. The reporting period represents a time of low pneumonia incidence in the San Francisco area; nevertheless, 447 cases were observed in the 85,142 enrollees over the age of 17 years, giving an annual rate of 9.1/1000. The proportion of pneumonia cases categorized as pneumococcal was 23% (103/447). Of these 103 cases of pneumococcal pneumonia, 91 (88%) were identified as serotypes that would be present in the multivalent pneumococcal vaccine.

The contractor has been successful in two developmental tasks: 1) establishing an epidemiological network which would be able to identify cases of pneumonia in the enrolled population; and 2) structuring a laboratory capable of securing specimens from these cases and properly culturing and serotyping pneumococci from them.

It is necessary to extend disease surveillance through the full 1972-1973 respiratory season to document a firm incidence rate. This information would be essential prior to a decision on suitability of this population for a vaccine trial.

Significance to NIAID Programs and Bio-Medical Research: Currently there is much interest in the prevalence of pneumococcal disease in a large open population. There is preliminary evidence to suggest subscribers of this medical group experience a relatively high incidence of disease. The program is sponsoring an intensive epidemiological study of pneumococcal disease in this population with ultimate aim of conducting a large field trial to show efficacy of a pneumococcal polyvalent vaccine.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at the end of the contract year.

Date Contract Initiated: March 1, 1972

Current Annual Level: $56,825 (for 6 months)

UNIVERSITY OF KENTUCKY (NIH 70-2265)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: Abram S. Benenson, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: A collaborative study to compare reactivity and immunogenicity of four smallpox vaccines (calf lymph, CAM, CV-1, and Elstree strains of vaccinia) administered at $10^6$, $10^7$, and $10^8$ pkfu percutaneously and $10^3$, $10^4$, and $10^5$ pkfu subcutaneously is in progress. These vaccinations were all followed after approximately six months by percutaneous vaccination with calf lymph vaccine at $10^8$ pkfu. Although very little of the post-challenge antibody data is available, the CV-1 vaccine appears to be less effective in inducing antibody formation than the other three vaccines. It appears that fewer complications are associated with subcutaneous vaccination and that fewer complications follow standard percutaneous inoculation in individuals previously given subcutaneous vaccine, although the same percentage of complications are seen in those who were previously given percutaneous vaccine.

Significance to NIAID Programs & Bio-Medical Research: At the present time routine smallpox immunization are not being given in the United States. Outbreaks of smallpox continue to occur in other countries. It is hoped that the present collaborative study will result in information that can be used to determine a better immunization procedure - for use whenever persons need to be vaccinated.

Proposed Course of Project: Contract to terminate on June 30, 1973

Date Contract Initiated: June 1970

Current Annual Level: $17,300 (6 months)

LEE-MAC - (NIH 72-2528)

Title: Production of Interferon in a Unique Cell Culture System

Contractor's Project Director: Thomas McCoy, Ph.D.

Project Officers: George J. Galasso, Ph.D.; Hilton Levy, Ph.D.

Objectives: If exogenous interferon proves to be effective in prophylactic and/or therapeutic use in man, an economically feasible method of producing interferon is needed. During the first year of this contract a feasibility study was performed to determine whether a unique cytogenerator could be used for this purpose. This having been accomplished, the goal for the second year was to determine whether interferon would be induced in this system.

Major Findings: A variety of alterations of media, pH, and manipulation of the cytogenerator have been successful in demonstrating the feasibility of producing large masses of diploid cells in this system. These studies have been successful in obtaining $3 \times 10^8$ viable cells in a volume of 200 milliliters. This is equivalent to the amount of cells produced in 75 completely confluent 250 ml tissue culture flasks. Studies on interferon induction have been done with chemical inducers such as polyinosinic·polycytidylic acid and viral inducers such as Newcastle disease virus. Many problems had to be overcome. This cytogenerator system is in very delicate balance, and pH changes caused by the induction system necessitated studies with new media and new buffers. These have now been overcome and the investigator has shown that interferon can be produced. The problem now remains on how to maximize the yield.

Significance to NIAID Programs and Biomedical Research: Interferon remains the most promising antiviral currently available for study toward feasibility for use in man. Two methods of administration are available: exogenous, prepared in vitro, and endogenous, through use of inducers. There is currently no successful method of economically producing large quantities of human interferon produced in cells other than human leucocytes. This contract is an attempt to determine whether a unique cytogenerator can overcome this obstacle. As indicated, this contractor has been successful in the two preliminary tasks set, i.e., produce large quantities of diploid cells and show that interferon can be produced in the system. The remaining task is to determine whether large yields are possible.

Proposed Course of Project: To be reviewed and results evaluated. If the cytogenerator is producing large quantities of interferon, attempts will be made to scale up. Because this is a fixed price contract, it must be terminated in June 1973. A new contract will be initiated if subsequent work is to be done.

Date Contract Initiated: June 1971

Current Annual Level: $52,000

MELOY LABORATORIES (NIH 71-2079)

Title: Maintenance of animal holding facility

Contractor's Project Director: R. W. Pasquale

Project Officers: Robert H. Purcell, M.D. and June K. Dunnick, Ph. D.

Objectives: To provide an animal holding facility, under isolated conditions, for the study of hepatitis in various primates, chiefly the rhesus monkey; to assist the project officer with animal observations, autopsies, collection of tissue specimens and histopathology.

Major Findings: The contractor has provided an excellent animal holding facility which has enabled the NIAID Hepatitis Program to characterize the agent of hepatitis B in primates. The rhesus monkey has been infected with the adw subtype of hepatitis B. The adw rhesus pool has been shown to be stable for eight months at -140°C, and unstable when treated at 100°C for 10 minutes, 80°C for one hour, or 60°C for one hour. Chloroform treatment inactivates the adw rhesus pool. The adw pool is infectious when administered intramuscularly or intravenously, but it is not infectious for rhesus monkeys when given orally. There is an excellent correlation between antigenemia and infectivity of serial serum samples from the rhesus monkey; i.e., material positive for HB Ag is infectious while HB Ag negative serum samples obtained from rhesus monkeys in the incubation or convalescent period are not infectious. It has further been demonstrated that if an animal develops an antibody response (HB Ab), he is immune to further challenge with HB Ag positive material.

Studies with baboons, pig-tailed macaques and woolly monkeys show that probably the latter can also serve as an animal model for hepatitis B infection. The baboon and pig-tailed macaque are not susceptible to hepatitis B infection. These studies have clearly demonstrated that primates can be used as animal models for human hepatitis B infection.

Significance to NIAID Program and Bio-Medical Research: These animal model studies are a necessary and integral part of the NIAID Collaborative Hepatitis Project, as they will yield basic knowledge on the characteristics of the agent of hepatitis B.

Proposed Course of Project: The contract has been renewed through December 31, 1973, at which time it will be reviewed for additional funding based on progress and programatic need.

Date Contract Initiated: June 23, 1967

Current Annual Level: $192,413

MOUNT SINAI SCHOOL OF MEDICINE (72-C-1181)

Title: Arrange and Conduct three Conferences on Influenza

Contractor's Project Director: Edwin D. Kilbourne, M.D.

Project Officer: Martha J. Mattheis

Objectives: To arrange and conduct a series of conferences (about 3 per year) on the genetics of influenza virus and new approaches to the control of influenza.

Major Findings: Three workshops entitled "Influenza Virus Polypeptides and Antigens", "Immunologic Methodology in Influenza Diagnosis", and "Specific Immunity in Influenza" were held during the first contract year. Two workshops entitled "Epidemiology of Influenza" and "Influenza Vaccines" were held during the current contract year. Two workshops entitled "Animal models for Influenza Research and Importance of Animals in Human Pandemic Influenza", and "Influenza Genetics, Viral Replication, and Replication Inhibition", are planned for the new contract year.

Summaries of these workshops are being published in the Journal of Infectious Diseases.

Significance to NIAID Program and Bio-Medical Research: It is predicted that another pandemic of influenza will occur during the period 1978-1980. This prediction is based on the recent past in which major mutations occurred in 1946, 1957, and 1968. This series of conferences will have the objective of developing ideas and directions for research in influenza which will contribute to development of NIAID programs.

Proposed Course of Project: If additional workshops are desired, this contract will be renewed.

Date Contract Initiated: June 30, 1971

Current Annual Level: $53,800 (estimate)

UNIVERSITY OF NEW MEXICO (NIH 71-2202)

Title: Epidemiology of pneumococcal pneumonia among the Navajo Indians

Contractor's Project Director: Betty Eberle, Ph. D.

Project Officer: Richard E. Horton, M.D.

Objectives: Complete epidemiological studies to determine the incidence of pneumococcal disease (pneumonia, otitis media and meningitis) in the Navajo Indian population receiving medical health care at the Gallup PHS Indian Hospital, New Mexico.

Major Findings: This collaborative project with the Indian Health Service (Project Director, Dr. George Bock) has completed a feasibility study to determine if the population within the geographic area served by the Gallup Service Unit would be suitable for a double-blind pneumococcal vaccine trial to demonstrate efficacy. Results from this study suggests this subsample of the Navajo Indian reservation has an exceedingly high incidence of pneumococcal disease (20 cases/1000/year) and the surveillance network established by the epidemiological team provides conditions for a satisfactory vaccine field trial.

Significance to NIAID Program and Bio-Medical Research: These studies have established a firm incidence rate of pneumococcal disease in a well-defined population base. This target population with a high frequency of pneumococcal pneumonia has great potential for a large field trial to demonstrate efficacy of a pneumococcal polyvalent vaccine.

Proposed Course of Project: This contract will be terminated at the end of the contract period, May, 1973.

Date Contract Initiated: June, 1971

Current Annual Level: 0

NEW YORK MEDICAL COLLEGE (PH43-68-1266)

Title: Therapeutic Use of Inducers of Interferon for Viral Infections of Animal and Human Eyes

Contractor's Project Director: Miles A. Galin, M.D.

Project Officers: Samuel Baron, M.D. and George J. Galasso, Ph. D.

Objectives: This is a study to evaluate the value of polyinosinic · polycytidylic acid, an inducer of interferon, in the treatment of viral diseases of the human eye, primarily herpetic keratoconjunctivitis.

Major Findings: The first phase of the project consisted of the evaluation of ocular and systemic toxicity of Poly (In·Cn) in six blind volunteers treated as in-hospital patients with a total dose of 4,900 micrograms by topical administration. There were no ocular toxic reactions or systemic toxicity as demonstrated by a series of blood studies taken prior to, during and after therapy.

Six patients who had been subject to recurrent herpetic keratoconjunctivitis were evaluated for toxicity in In·Cn when they were in a non-infected state. All ocular and laboratory tests done in the blind controls were repeated in these patients. No toxicity was found.

The patients in the double-blind study in In·Cn or IDU (A or B) were divided into three diagnostic groups (I) acute herpetic keratoconjunctivitis, treated with In·Cn or IDU; (II) chronic recurrent herpetic keratoconjunctivitis with epithelial lesions, treated with In·Cn or IDU; (III) chronic recurrent herpetic keratoconjunctivitis without epithelial lesions, treated with In·Cn or artificial tears (Tearisol) (C or D). Patients in Group I responded well. Patients in Group II became worse. Most patients in Group III had no recurrences. It would appear that treatment of chronic keratoconjunctivitis is ineffective. The role of IDU and In·Cn in the treatment of acute cases of herpetic keratoconjunctivitis still remains to be determined because of the relatively few numbers of acute patients treated. There is insufficient data to fully evaluate the efficacy of In·Cn.

Significance to NIAID Programs and Bio-Medical Research: Polyinosinic · polycytidylic acid is an effective inducer of interferon. This topical treatment, if effective, would solve an important medical disease problem which is common and very painful to the patient. The current therapy, although useful, needs improvement.

Proposed Course of Project: To terminate on March 19, 1974.

Date Contract Initiated: June 20, 1968

Current Annual Level: $44,273 (9 months)

STATE UNIVERSITY OF NEW YORK AT BUFFALO

Title: Hepatitis Study Center

Contractor's Project Director: Pearay L. Ogra, M.D.

Project Officers: June K. Dunnick, Ph. D., Robert H. Purcell, M.D., and David J. Gocke, M.D.

Objectives: Perform an epidemiologic survey of hepatitis B in a population in which viral hepatitis is highly endemic. The study will include identification of infecting source, method and prevalence of spread, identification of hepatitis B subtypes and incubation time of the disease. Emphasis should be placed on a study of non-parenteral transmission of hepatitis B.

Major Findings: As the contract will not be initiated until May 1973, there are no major findings.

Significance to NIAID Program and Bio-Medical Research: A Hepatitis Study Center would be an integral part of the NIAID Hepatitis Program. This center would serve as a basis for obtaining information on the epidemiology of hepatitis B infection and the bioimmunology of this disease. If the population selected for study would benefit by immunization procedures, the NIAID plans to study the effectiveness of hepatitis B antibody containing gamma globulin in preventing the disease.

Proposed Course of Project: It is estimated that it will take two years to complete the objective of this contract.

Date Contract Initiated: May 1973

Current Annual Level: $91,600

NEW YORK UNIVERSITY MEDICAL CENTER (NIH 69-2222)

Title: Longitudinal Evaluation of Vaccines for the Control of Rubella

Contractor's Project Director: Louis Z. Cooper, M.D.

Project Officer: Martha J. Mattheis

Objectives: To conduct: 1) longitudinal studies of vaccine efficacy and safety, 2) studies of unattenuated (natural) rubella and congenital rubella and 3) to develop improved techniques for characterizing the immunology of rubella infection.

Major Findings: Longitudinal studies of HI antibody persistence after rubella, rubella vaccination and congenital rubella among persons living in the community and in an institution which has been "free from detectable rubella" since 1964 have revealed no change in the pattern described previously. That is, the rate of decline of HI titers following rubella or rubella vaccination is greater among the institutionalized population. In congenital rubella, decline is similar in both populations. The presence of rubella specific IgM HI antibody was correlated with young age, virus shedding and elevated levels of total IgM in a group of 143 children studied during the first two years of life. Ongoing comprehensive rubella antibody screening and vaccination programs have continued in Bellevue and University Hospitals' Employee Health Clinics, the Bellevue-Hunter Student Nurse Program and the Prenatal Clinics. The susceptibility rate for this young adult population is approximately 12% with the exception of those groups which are predominately Puerto Rican, where susceptibility exceeds 20%. Vaccination with HPV-77DE$_5$, Cendehill or RA27/3 has been uneventful in these young adults with joint reactions generally mild and uncommon.

Significance to NIAID Program and Bio-Medical Research: This contract will answer a number of questions concerning immunity following natural infection and immunity following vaccination. The widespread utilization of rubella vaccines makes it mandatory to assure the continued protective efficacy and safety of these vaccines.

Proposed Course of Project: Surveillance studies will be continued until June, 1974.

Date Contract Initiated: June, 1969

Current Annual Level: (Prior year support)

NEW YORK UNIVERSITY MEDICAL CENTER - (NIH 70-2131)

Title: The Evaluation of Topical, Intralesional and Systemic Interferon and Interferon Inducers in the Prevention and Treatment of the Fibroma Virus-Induced Skin Lesion in Rabbits

Contractor's Project Director: Alvin E. Friedman-Kien, M.D.

Project Officers: George J. Galasso, Ph.D.; Samuel Baron, M.D.

Objectives: To provide an animal-model system which closely approximates a disease entity in man for the purpose of examining antivirals as they become available and to assess their potential value. This particular system is for viral diseases of the skin.

Major Findings: This model provides us with lesions that can be easily observed and used for the systemic, topical and intralesional administration of antiviral agents. It also has developed a herpetic keratoconjunctivitis model in the rabbit for further topical evaluation of potential antivirals.

During the previous contract periods, interferon and the potent inducer of interferon, polyinosinic·polycytidylic acid (poly I·poly C), were found to be extremely effective in suppressing the RFV and VV lesions when given systemically or intralesionally prior to the actual appearance of the lesions. The contractor has recently been able to produce concentrated rabbit interferon in vitro so that a higher titer of interferon can be delivered to the site of infection with excellent antiviral results. This material has been administered topically and by subconjunctival injection and has provided excellent protection in HSV-infected rabbit corneas. Further studies are in progress in which concentrated interferon is given after the lesion has developed, to obtain a therapeutic rather than a prophylactic effect.

Ara-A given systemically in doses of 300 mg/kilo or 600 mg/kilo showed an excellent antiviral effect against both the RFV and VV skin infections. A suppression of the RFV lesion was observed even when treatment began after the lesion developed. Further testing of the therapeutic efficacy of Ara-A is now in progress.

Significance to NIAID Programs and Biomedical Research: Having firmly established baseline data on the effects of interferon and interferon inducers in these animal models, the program will be better able to directly test new antivirals as they are developed in the selected systems. This will afford the program with a direct means of comparison in determining the relative merits of the various substances and their potential for clinical use.

Proposed Course of Project: It is anticipated that this project will be continued through June 1974 when it will be re-evaluated, with continued support based on programmatic need.

Date Contract Initiated: May 25, 1970

Current Annual Level: $65,503

NEW YORK UNIVERSITY MEDICAL CENTER - (NIH-70-2169)

Title: Methods for the Production of Human Interferon

Contractor's Project Director: Jan T. Vilcek, M.D.

Project Officers: George J. Galasso, Ph.D.; Samuel Baron, M.D.

Objectives: Develop a technique for a more efficient, safe and economically feasible production of human interferon. A large number of commercially available cells will be investigated for this purpose. Clones will be obtained from the more promising cells and the enhancing effect of anti-metabolites studied. The contractor will concentrate on manipulation of inducers and inducing factors in order to increase the yield of the interferon to practical levels. This contractor will also provide 100 million units of human diploid cell interferon for purification studies.

Major Findings: Dr. Vilcek has generated much information in fulfilling the objectives of the workscope by exploring possible methods of optimizing human interferon production by using enhancers, super-induction, priming, and regulation of variables in the tissue culture medium. By these techniques he has been able to increase the yield approximately 10-fold for each contract year. If he can achieve another 10-fold increase and these techniques are adapted to large-scale production, sufficient quantities will be available for clinical use at a realistic cost.

Work during the past year has shown that suitable priming with interferon produced on average an about 8-fold increase over the control yield. Under the optimal conditions, super-induction produced an about 100-fold increase over the average control yield. Combined super-induction and priming did not produce yields markedly higher than obtainable by super-induction alone. Essentially similar results were obtained in cultures of human embryonic kidney cells and in FS-3 cells stimulated with other double-stranded polynucleotide inducers. Screening of cell strains derived from individual neonate foreskins resulted in the selection of a high-producer strain (FS-4) which is currently employed in all studies. Current yields from stationary cultures are in the range of about 40,000 reference units/2 ml from cultures containing about $1 \times 10^6$ cells. Experimental production in roller bottle cultures has been started. The stability of interferon activity on storage appears to be satisfactory.

Significance to NIAID Programs and Biomedical Research: All the interferon inducers currently available are toxic; therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case, a more efficient and economically feasible means of producing interferon must be found.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: May 25, 1970

Current Annual Level: $61,438

THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL (NIH 72-2505)

Title: Clinical evaluation of respiratory vaccine in children and infants

Contractor's Project Director: Floyd W. Denny, Jr., M.D.

Project Officers: Robert M. Chanock, M.D. and June K. Dunnick, Ph. D.

Objectives: The contractor will conduct clinical evaluation of live virus vaccines with a major emphasis on the evaluation of live respiratory syncytial virus (RS) vaccine in a population of infants to ascertain infectivity, lack of virulence and production of antibody; and in semi-closed or carefully monitored open populations of children and infants to obtain additional information on infectivity, degree of virulence, production of antibody and transmissibility.

If the need should arise, and at the direction of the Project Officer, the contractor will conduct clinical studies to evaluate reactivity and antigenicity of candidate bacterial polysaccharide vaccines, specifically Hemophilus influenzae type b polyribose phosphate vaccine.

Major Findings: The contractor has performed a trial to evaluate the RS-A2 ts-1 mutant viral vaccine, testing the safety, antigenicity, infectivity, immunogenicity and person-to-person spread of this vaccine. The vaccine was administered to four one-year-olds without pre-existing antibody, and all four children were successfully infected; the degree of immunity conferred is yet to be determined. The contractor continues to survey his population for natural RS virus infection and other respiratory infections.

Significance to NIAID Program and Bio-Medical Research: This contract is part of the schedule of the respiratory virus vaccine development program; it is necessary to do careful clinical trials on vaccines that are likely to be effective in reducing respiratory illnesses.

Proposed Course of Project: The contract has been funded through March 31, 1974, at which time it will be reviewed for additional funding based on progress and programatic need.

Date Contract Initiated: April 1, 1972

Current Annual Level: $47,102

OHIO STATE UNIVERSITY (PH43-67-78)

Title: Influence of cultural condition and concentration procedures on the antigenicity of Mycoplasma pneumoniae vaccines

Contractor's Project Director: Norman Somerson, Ph. D.

Project Officers: Robert Purcell, M.D., Martha Mattheis

Objectives: 1) Produce 20,000 ml of an experimental M. pneumoniae vaccine, using strain C18 2) develop a process suitable for large-scale commercial production of vaccine.

Major Findings: The two lots of vaccine totaling 20,000 doses have been prepared, safety tested, packaged, and shipped to NIH for use in vaccine trials. These vaccines contain 260 ug/ml protein as determined by the Lowry method, which is two times as much protein as was present in the best lot prepared up to this time. They are 10-fold more antigenic in animals than the best lot that was prepared prior to this. All of the 13 vaccinees who received the vaccine developed a 4-fold or greater rise in antibody titer to M. pneumoniae.

This manufacturing process is commercially feasible if the active component of the serum fraction used in the growth process can be determined. This contractor is attempting to define that component.

Significance to NIAID Programs and Bio-Medical Research: Twenty thousand doses of inactivated M. pneumoniae vaccine are available for evaluation for efficacy in a field trial. The question of whether or not an inactivated M. pneumoniae vaccine will be effective in preventing disease due to M. pneumoniae can now be answered.

Proposed Course of Project: This contract will terminate May 31, 1973.

Date Contract Initiated: December, 1966

Current Annual Level: $12,000

MEDICAL COLLEGE OF PENNSYLVANIA - (NIH-70-2126)

Title: Production of Anti-Interferon Sera and Development of Immunoassays

Contractor's Project Director: Kurt Paucker, Ph.D.

Project Officers: George J. Galasso, Ph.D.; Samuel Baron, M.D.

Objectives: Apply the techniques developed with mouse interferon to produce anti-human interferon sera. This material will be sufficiently specific to inhibit the antiviral effect of interferon. This material will be extremely useful, not only for studies on mechanism of action of interferon but also for affinity chromatography studies on purification of interferon.

Major Findings: Dr. Paucker has successfully produced high-titered anti-mouse interferon serum in rabbits. The antigens used to immunize rabbits included both crude and partially purified interferon induced in L cells with UV-irradiated NDV and crude interferon produced in the same cell line using poly I·poly C as the stimulus. Appropriate normal cell antigens were also used to immunize rabbits in order to test the specificity of the antibodies produced against interferon-containing materials.

The mouse interferon antiserum proved to be specific, and the antisera was made more specific by adsorption with antigens derived from the cell system used to produce the interferon antigen. Antibodies to these extraneous proteins were completely eliminated from the anti-mouse interferon globulin without diminution of neutralizing titer. Approximately 100 ml of adsorbed anti-mouse interferon globulin packaged in 1- and 5-ml amounts were delivered to the NIAID for distribution; several hundred ml of non-adsorbed serum are in stock and await processing.

During the past contract year the emphasis has switched to producing anti-human interferon globulin. Human interferon was prepared to the extent of $12 \times 10^6$ units in U amnion and $6 \times 10^6$ units in embryonic skin cells. Currently four rabbits are undergoing immunization with human interferon and others are being added to the program.

Significance to NIAID Programs and Biomedical Research: Specific antisera to interferon can be used to develop an immunoassay and, more important, in studies on mechanisms of interferon induction. When an inhibitory reaction is detected, the antisera can be used to determine whether the reaction is due to interferon or some other inhibitory effect.

Proposed Course of Project: To be reviewed and the results evaluated and considered for renewal at the end of the contract year.

Date Contract Initiated: June 1, 1970

Current Annual Level: $44,601

TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA (PH43-67-681)

Title: Epidemiology of pneumococcal disease in preparation for field trials with pneumococcal vaccine

Contractor's Project Director: Robert Austrian, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: The major emphasis of the contract will be a two-year program to conduct studies on the epidemiology and immunology of pneumococcal infection in conjunction with controlled field trials to demonstrate efficacy of candidate multivalent pneumococcal vaccines in selected populations.

1. Serve as central facility for the collection and processing of pertinent epidemiologic data submitted from the following instutitions participating in the NIAID pneumococcal vaccine program: a. Dorothea Dix Hospital, Raleigh, North Carolina, b. Kaiser Permanente Medical Group, San Francisco, California, c. Navajo Indian Health Service, Gallup, New Mexico.

2. Continue to direct and coordinate clinical and laboratory surveillance activities at Dorothea Dix, Raleigh, North Carolina, during a controlled vaccine field trial to be initiated in January, 1973.

3. Continue to serve as a reference serological laboratory for the radioimmunoassay to assess the human antibody response to natural pneumococcal infections and active immunization with purified capsular polysaccharides.

Major Findings: Testing of monovalent and of polyvalent vaccines of pneumococcal capsular polysaccharides prepared by Eli Lilly and Co., both for immunogenicity and reactivity has been prosecuted during the contract period. Five hundred thirty-nine immunizations were performed, including 299 with monovalent vaccines, 104 with hexavalent vaccine I, containing the capsular polysaccharides of types 1, 3, 4, 7, 8 and 12; 67 with hexavalent vaccine II, containing the polysaccharides of types 2, 5, 6, 14, 18 and 19; and 69 with dodecavalent vaccines containing the 12 polysaccharides included in the two hexavalent vaccines. Methods for assaying antigenicity in terms of immunologic reactivity in vitro, of monovalent and of the individual components of polyvalent vaccines have been developed by modification of the radioimmunoassay.

The antigenicity of monovalent preparations of the polysaccharides of the following pneumococcal types has now been tested in man: types 1-9, 12, 14, 18, 19 and 23. All have been given in doses of 50 ug and 100 ug and many have been tested in doses of 12.5 ug, 25 ug, 500 ug and 1,000 ug as well. All monovalent preparations and all components of polyvalent preparations have been shown to be antigenic.

Sequential immunization with several monovalent vaccines has been carried out in a number of individuals. Assay of their sera has demonstrated clearly that initial immunization with one polysaccharide does not interfere with subsequent

TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA (PH43-67-681) - continued

immunization with one or more heterologous polysaccharides and that later immunization does not augment or depress the pre-existing level of antibody resulting from one or more previous immunizations. No evidence of interference among antigens in polyvalent vaccines has been demonstrable.

A field trial of two polyvalent vaccines has been initiated at Dorothea Dix Hospital in January 1973, following approval of the protocol by committees reviewing the use of human subjects in research at Dorothea Dix Hospital, the University of Pennsylvania and the National Institutes of Health. The study is being conducted in "double-blind" fashion.

Additional data regarding the pneumococcal types responsible for otitis media have been obtained from strains isolated from the middle ear by Dr. Virgil M. Howie of Huntsville, Alabama. Since type surveillance was begun in February 1970, 415 isolates from 344 patients have been identified. The types (Danish classification) recovered most often in rank order of frequency are 19, 23, 6, 14, 3, 18, 1 and 4. These eight types account for 73.7% of all otitic infection attributable to pneumococcus in this sample.

The age distribution of acute otitis media indicates 48.6% occurred in the first year of life and 26.8% in the second year of life. Of 56 children less than two years of age whose sera have been assayed, only 10 (17.8%) showed an antibody response to the pneumococcal serotype isolated, whereas among the sera of 27 patients two years of age or older, 13 (48.1%) had a serologic response to infection. The response to infection is in general accord with the age-related response to several polysaccharide vaccines described by others.

Significance to NIAID Program and Bio-Medical Research: Epidemiological analysis of clinical and immunological data will aid in the interpretation of vaccine field trials to demonstrate efficacy of newly developed pneumococcal vaccines.

Proposed Course of Project: The contract will be reviewed and evaluated for renewal at the end of the contract year, May 1974.

Date Contract Initiated: February 1967

Current Annual Level: $290,000

UNIVERSITY OF PITTSBURGH - (NIH-72-2523)

Title: Biological Stimulators of Interferon

Contractor's Project Director: Julius S. Youngner, Sc.D.

Project Officers: George J. Galasso, Ph.D.; Samuel Baron, M.D.

Objectives: All attempts to date to yield a nontoxic interferon stimulator, whether biologically or artificially derived, have been unsuccessful. The effective dosage of interferon may be so high as to render the exogenous route prohibitive for many applications. There is therefore a need to develop an inducer which is both nontoxic and highly effective. An efficacious approach to this search may be an investigation of biological products. This is one of two contracts to determine which of the available biological inducers have clinical potential.

Major Findings: Several interferon stimuli will be evaluated, including cell wall products of S. minnesota strains, Brucella abortus strains, and certain fungi. There are three projected phases of the work, including (1) evaluation of interferon production and toxicity in mice and rabbits, (2) testing of efficacy in animal model systems, and (3) identification of the active chemical moiety involved in interferon induction.

Progress has been made in the production, chemical and biological characterization of the glycolipid (GLP) of S. minnesota strain R 595. Production of the lipopolysaccharide from Xanthomonas sp., a pigmented plant pathogen, has been undertaken. Brucella abortus cells extracted with diethyl ether have been shown to be excellent interferon stimuli in mice by various routes. This latter inducer, in contrast to GLP, produces a "virus type" interferon response. A series of purified yeast polysaccharides have undergone initial screening.

Significance to NIAID Programs and Biomedical Research: Interferon holds great promise for clinical application. This can be achieved through exogenous administration or endogenous production through use of safe interferon inducers. This project is an attempt to obtain an inducer of biological origin which will prove effective and safe with the ultimate goal of use in man.

Proposed Course of Project: This contract will be continued through June 1976 with three years of incremental funding: the first phase for the acquisition, production and purification of selected biological inducers, the second phase for tissue culture and animal studies to determine the most effective inducers, and the final phase to determine the actual moiety of the inducer responsible for the interferon stimulation.

Date Contract Initiated: June 26, 1972

Current Annual Level: $73,025 (estimated)

UNIVERSITY OF ROCHESTER (NIH 72-2503)

Title: Conduct clinical evaluation of respiratory viral and bacterial vaccines in children and infants

Contractor's Project Director: Gordon Douglas, Jr., M.D.

Project Officers: Richard E. Horton, M.D. and Brian Murphy, M.D.

Objectives: Clinical evaluation of live attenuated virus vaccines (RSV and influenza) and inactivated vaccines (Mycoplasma pneumoniae, adenovirus and parainfluenza virus). The major effort to be placed on testing respiratory syncytial virus vaccine in closed populations of infants to ascertain infectivity, degree of virulence and production of antibody.

Clinical studies to evaluate reactivity and antigenicity of candidate bacterial polysaccharides with major emphasis on Hemophilus influenzae type b polyribose phosphate (PRP) vaccine in children.

Major Findings: This contract was initiated June 1, 1972. During the first contract period, four children were studied after receiving RSV vaccine, 21 young adults and 36 elderly were immunized intranasally with live influenza virus vaccine and studied; and 15 children were vaccinated with a single dose of H. influenzae type b PRP vaccine. A natural outbreak of RSV infection occurred and curtailed further RSV trials for the past winter. Studies with respiratory syncytial virus vaccines have indicated that more attenuated vaccine strains are required; the contractor will continue to study reduced dose and method of inoculation with the presently available vaccine strain.

Studies with live attenuated influenza virus vaccines will concentrate mainly on evaluating strains carrying the most recent influenza antigens. It will first be tested in normal young adults and then in elderly healthy subjects before going to elderly chronically ill subjects. Pneumococcal polysaccharide vaccine studies will be conducted in elderly subjects. Other vaccines such as adenovirus subunit vaccines, and inactivated Mycoplasma pneumoniae vaccines can be studied.

Significance to NIAID Program and Bio-Medical Research: The data obtained from these studies will help to establish the safety and antigenicity of new prototype respiratory viral and Mycoplasma pneumoniae vaccines being developed by the Infectious Disease Branch. Studies with H. influenzae (PRP) vaccine will aid immensely in developing an optimal immunization schedule for this vaccine in young children and infants.

Proposed Course of Project: To be reviewed and evaluated for renewal at the end of the contract year.

Date Contract Initiated: May, 1972

Current Annual Level: $130,005

ROCKEFELLER UNIVERSITY (NIH 71-2502)

Title: Radioimmunoassay for the quantitative assay of human anti-meningococcal antibodies

Contractor's Project Director: Emil Gotschlich, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: Using a radioimmunoassay procedure, serve as a reference testing laboratory to measure the serum antibody response of children and infants that have been immunized with group A and group C meningococcal polysaccharide vaccines. Contractor should be prepared to process at least 150 specimens per month, including paired sera from subjects convalescing from natural illness.

Major Findings: In the second contract period, the test employing the C antigen has been perfected. The following methodology has been employed. Highly purified group C antigen was further purified by digestion at pH 7.5 for 24 hours with insoluble proteolytic enzyme obtained commercially from Miles under the trade name Enzite Protease. The polysaccharide was separated from the cellulose bound enzyme by filtration, and was then dialyzed extensively to remove split products. The dialyzate was passed over a cationic ion exchanger SE Sephadex and was then precipitated with ethanol and acetone dried. Fifty mg of this polysaccharide was activated for 5 minutes at pH 9.0 with 20 mg of cyanuric chloride at room temperature and was then allowed to couple overnight at pH 7.0 to tyramine. The polysaccharide was then purified by dialysis and chromatography over SE Sephadex, and was recovered by alcohol precipitation and acetone drying. This material readily iodinates accepting approximately 58% of added radioactive iodine. In a fresh preparation approximately 85% of the radioactivity is precipitable by immune serum, whereas less than 6% is bound by fetal calf serum.

The same methodology has been used with group B polysaccharide. The results are nearly identical in that approximately 85% is precipitable by rabbit hyperimmune serum and less than 10% is bound to fetal calf serum. However, normal sera bind approximately 50% of this antigen, irrespective of the antigen concentration. The binding is somewhat less pronounced in the sera of very young children but still quite high. Efforts have been made to determine whether the binding is specific or non-specific. Two findings suggest that it is the gamma globulins in the serum which are binding the antigen. First, when goat anti-human immunoglobulin antiserum is substituted for ammonium sulfate to precipitate the immunoglobulins in the human serum, the results are identical. In addition, if sera from patients with agammaglobulinemia are studied, markedly less binding of antigen is found. The next question is whether the binding to the gamma globulin was specific or unspecific. As a first experiment in this direction, the contractor measured the binding capacity of three myeloma proteins of the IgG, IgM and IgA class. None of these exhibited significant binding. These findings, while not conclusive, suggest that antibodies to the group B polysaccharide are prevalent.

ROCKEFELLER UNIVERSITY (NIH 71-2502) - continued

Significance to NIAID Program and Bio-Medical Research: There is a need to develop a highly sensitive and quantitative serological procedure to measure low levels of antibody in infants and children recovering from meningococcal meningitis and those who have been actively immunized with purified meningo-polysaccharides. Combined development of the RIA procedure has the potential of achieving this goal. The services of a reference serological laboratory is essential for a comprehensive assessment of the antigenic and protective capabilities of meningococcal vaccine for children and infants.

Proposed Course of Project: This contract will be reviewed and evaluated before the anniversary date, May, 1973.

Date Contract Initiated: June, 1971

Current Annual Level: $24,760 (estimated)

RUTGERS UNIVERSITY - (NIH 72-2504)

Title: Development of Attenuated Mutants of Respiratory Syncytial Virus and Parainfluenza viruses as Potential Vaccine Strains

Contractor's Project Director: Robert W. Simpson, Ph. D.

Project Officer: Robert M. Chanock, M.D. and Martha J. Mattheis

Objectives: By the use of mutagenic agents, and/or viral recombination, and/or other methods, prepare and evaluate new viral strains of respiratory syncytial virus and parainfluenza viruses, types 3, 1 and 2 with characteristics suitable for use as potential vaccine strains. The attenuated viruses should be infective, less virulent than the parent virus, and induce local and/or serum neutralizing antibody in appropriate test animals, e.g. hamsters.

Major Findings: Most of the objectives outlined in the original proposal for the first quarter have been accomplished. The A-2 strain of RS virus was worked with exclusively during this period. It has been propagated in primary bovine embryonic kidney, human embryonic kidney and Hep-2 cells. Pools of RS virus prepared ir the latter cells are being utilized only for biochemical studies. The best plaque assays have been obtained with the Hep-2 cells which give highly reproducible results and remain viable for many days under solid overlay, unlike other cell types tested.

Significance to NIAID Program and Bio-Medical Research: Respiratory syncytial virus (RSV) and parainfluenza type 3 virus cause a highly significant amount of severe illness in young children. A safe, effective vaccine to these viruses is of the highest priority.

Proposed Course of Project: Renewal is anticipated.

Date Contract Initiated: June 1, 1972

Current Annual Level: $71,000

ST. LOUIS UNIVERSITY SCHOOL OF MEDICINE (NIH 70-2266)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: James D. Cherry, M.D.

Project Officer: George J. Galasso, Ph. D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: A collaborative study to compare reactivity and immunogenicity of four smallpox vaccines (calf lymph, CAM, CV-1, and Elstree strains of vaccinia) administered at $10^6$, $10^7$, and $10^8$ pkfu percutaneously and $10^3$, $10^4$, and $10^5$ pkfu subcutaneously is in progress. These vaccinations were all followed after approximately six months by percutaneous vaccination with calf lymph vaccine at $10^8$ pkfu. Although very little of the post-challenge antibody data is available, the CV-1 vaccine appears to be less effective in inducing antibody formation than the other three vaccines. It appears that fewer complications are associated with subcutaneous vaccination and that fewer complications follow standard percutaneous inoculation in individuals previously given subcutaneous vaccine, although the same percentage of complications are seen in those who were previously given percutaneous vaccine.

Significance to NIAID Programs & Bio-Medical Research: At the present time routine smallpox immunization are not being given in the United States. Outbreaks of smallpox continue to occur in other countries. It is hoped that the present collaborative study will result in information that can be used to determine a better immunization procedure - for use whenever persons need to be vaccinated.

Proposed Course of Project: Contract to terminate June 11, 1973

Date Contract Initiated: June 12, 1970

Current Annual Level: $39,935 (6 months)

SCRIPPS CLINIC AND RESEARCH FOUNDATION (NIH 73-2509)

Title: Immunohistochemical assay of hepatitis virus associated antigens

Contractor's Project Director: Thomas S. Edgington, M.D.

Project Officer: June K. Dunnick, Ph. D.

Objectives: The contractor shall develop methods for immunohistochemical assays of intrahepatic serum hepatitis virus-associated antigens and do detection studies on primate liver biopsies as directed by the project officer.

Major Findings: The contractor has made immunohistochemical reagents for detecting hepatitis B antigens including the "a", "y" and "d" antigenic specificities. He is in the process of developing reagents for other hepatitis B antigens, including reagents for detecting DANE particles and core components. Assays for hepatitis B antigens are done on primate species.

Significance to NIAID Program and Bio-Medical Research: This contract is an important part of the overall NIAID Hepatitis Program. It serves to detect hepatitis B infection in animals used in the NIAID collaborative animal model studies. Immunohistochemical assay procedures represent one of the most sensitive techniques for detecting hepatitis B antigen in liver biopsy specimens.

Proposed Course of Project: The contract is for a period of one year; through October 31, 1973, at which time it will be reviewed for additional funding based on progress and programatic need.
Date Contract Initiated: November 1, 1972

Current Annual Level: $108,431

SMITH, KLINE AND FRENCH LABORATORIES - (NIH-72-2516)

Title: Partial Purification, Safety Testing, and Preparation of Concentrated Human Leucocyte Interferon for Clinical Trial

Contractor's Project Director: Richard F. Haff, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: The state of the art has now advanced to the point that exogenous interferon can be tested for efficacy in a clinical trial. The purpose of this contract is to process interferon (concentrate, partially purify, safety test and ampoule) for a clinical trial on varicella-zoster in the immunologically suppressed patient.

Major Findings: Exogenous human leucocyte interferon has been processed and has been made available to the clinical evaluation team. SKF has also provided samples of lyophilized interferon so that sudies can be made to determine whether future studies can be done using freeze-dried material which might prove more stable and easier to store.

Significance to NIAID Programs and Biomedical Research: One of the initial aims of the Antiviral Substances Program was and continues to be to determine whether exogenous interferon can be used efficaciously in man. This contract is an integral part of an attempt to test this material.

Proposed Course of Project: This contract was extended during FY 73 to provide additional interferon for another clinical study. It is not anticipated that it will continue next year; this will depend on program needs for this material.

Date Contract Initiated: June 1972

Current Annual Level: $34,536

UNIVERSITY OF SOUTHERN CALIFORNIA (NIH 72-2506)

Title: Evaluation of H. influenzae type b, and meningococcal (group A and C) vaccines in infants and children

Contractor's Project Director: Paul F. Wehrle, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: Direct and coordinate studies to determine optimal immunization schedules for H. influenzae type b PRP vaccine and group A and group C meningococcal vaccines in very young children (ages 1-3) and infants less than 12 months of age. Basic variables to be explored include: reactivity, antigenicity of each candidate vaccine by dose (polysaccharide concentration), route of administration, time interval between primary immunization and the booster effect accompanying a secondary immunization.

Major Findings: The major accomplishments of this project during the first contract year has resulted in 88 infants and children being immunized with H. influenzae type b (PRP) vaccine, 97 infants being immunized with group C meningococcal polysaccharide vaccine, and 27 infants vaccinated with group A meningococcal polysaccharide vaccine. The contractor reports no adverse local or systemic reactions associated with any of the three prototype vaccines undergoing clinical evaluation. Preliminary serological information based on radioimmunoassay (RIA) of antibody levels has demonstrated that the immune response of infants to a 50 ug dose of PRP vaccine is lower in magnitude than that of older children and adults. Serological response of recipients of group C and group A meningococcal vaccines are being analyzed by radio antigen binding procedure of Dr. Gotschlich. Studies are in progress to assess the duration of the primary immune response and the enhancing effect of individual booster doses of the homotypic polysaccharide.

Significance to NIAID Program and Bio-Medical Research: Epidemiological studies have shown the major portion of bacterial meningitis of young children and infants is due to H. influenzae and group A and C Neisseria meningitidis. If clinical studies show the young infant can respond satisfactorily to immunization with purified polysaccharide vaccines, it may be possible to reduce the prevalence of these serious infectious diseases of the central nervous system by an immunoprophylactic approach.

Proposed Course of Project: This contract will be reviewed and evaluated prior to April 1974 and decision made on renewal depending on program requirements.

Date Contract Initiated: April, 1972

Current Annual Level: $66,279

STANFORD UNIVERSITY - (NIH 71-2201)

Title: Methods of Increasing Resistance of Polyinosinic·Polycytidylic Acid to Human Serum Hydrolytic Enzymes

Contractor's Project Director: Thomas C. Merigan, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: To study chemical modifications of the chemical inducer polyinosinic·polycytidylic acid (poly I·poly C) in order to stabilize it against human serum hydrolytic enzymes. Ultimate goal is to obtain an effective, less toxic inducer for use in man.

Major Findings: A series of new chemical inducers which are modifications of poly I·poly C have been produced and tested in selected models by the contractor. Results have indicated that chemical modifications which resulted in a more potent interferon inducer were also more toxic in animal studies. Therefore, it appears that this approach is not a useful one in terms of obtaining a more efficient inducer for clinical studies.

Significance to NIAID Programs and Biomedical Research: Interferon remains the most promising antiviral currently available for study toward feasibility for use in man. Two methods of administration are available: exogenous, prepared in vitro; and endogenous, through use of inducers. The inducers available are somewhat toxic, including the most promising, poly I·poly C. This contract was an attempt to stabilize the compound so that the dosage could be reduced without affecting its inducing abilities and also to obtain a more effective inducer.

Proposed Course of Project: Terminated.

Date Contract Initiated: June 1971

Current Annual Level: Prior year funds.

STANFORD UNIVERSITY - (NIH-NIAID-73-2501)

Title: Evaluation of Therapeutic Efficacy of Human Interferon in Disseminated Varicella-Zoster Infections

Contractor's Project Director: Thomas C. Merigan, M.D.

Project Officers: Michael Worthington, M.D.; George J. Galasso, Ph.D.

Objectives: One of the primary goals of the Antiviral Substances Program/ NIAID is to help demonstrate whether interferon could be used efficaciously in man against viral disease. This project offers one opportunity to assess this feasibility.

Major Findings: Prime disease candidates for evaluation of clinical efficacy of exogenous interferon are those whose pathogenesis is dependent on continued viral replication. Disseminated herpes zoster infection of immunologically compromised patients offers such a model. An Investigative New Drug application has been submitted to the Food and Drug Administration, and studies are under way to determine the effectiveness of exogenous interferon in such patients. The initial months of the contract were spent in organizing the study and obtaining the material for clinical testing. It is too early to present results, especially since the study will be performed in a double-blind fashion using placebo.

Significance to NIAID Programs and Biomedical Research: This study is part of the ASP studies to determine whether or not exogenous human interferon can be used prophylactically or therapeutically in man.

Proposed Course of Project: This contract will probably continue through the option-to-renew mechanism for two additional years through December 1975 or January 1976.

Date Contract Initiated: January 1, 1973

Current Annual Level: $58,585

TULANE UNIVERSITY, DELTA REGIONAL PRIMATE RESEARCH CENTER (NIH 71-2197)

Title: Studies of response of sero-negative chimpanzees following exposure to hepatitis B antigen (HB Ag).

Contractor's Project Director: Dr. William P. Allen

Project Officers: Robert H. Purcell, M.D. and June K. Dunnick, Ph. D.

Objectives: To furnish healthy sero-negative chimpanzees and to provide for all necessary care of these animals, including veterinary care; these chimpanzees will be housed under isolated conditions for the duration of the experiments. The contractor will obtain blood samples, biopsy specimens, make clinical observations and furnish laboratory services.

Major Findings: The contractor has identified sero-negative chimpanzees suitable for hepatitis B studies; chimps used in hepatitis B studies are housed in isolated quarters. The first experiment to determine infectivity of various dilutions of a known infectious hepatitis B pool in the chimp was begun in February 1973. These studies are part of a collaborative National Institute of Allergy and Infectious Diseases/Food and Drug Administration/ Center for Disease Control hepatitis B animal model study.

Significance to NIAID Program and Bio-Medical Research: The chimpanzee has been infected with hepatitis B material by several workers. Dr. Barker, BoB, FDA, has demonstrated clinical or subclinical hepatitis in two of six chimpanzees inoculated with potentially infectious material. Thus, the chimpanzee is one of the few animals that has been shown to get clinical recognizable hepatitis B infection, and this information could eventually be used to test prospective hepatitis B vaccines.

Proposed Course of Project: The contract has been funded through March 20, 1974, at which time it will be reviewed for additional funding based on progress and programatic need.

Date Contract Initiated: June, 1971

Current Annual Level: $12,000

UNIVERSITY OF UTAH - (NIH-70-2175)

Title: Study of Interferon and Interferon Inducers in the Prevention of Rabies in Animals

Contractor's Project Director: Burton Janis, M.D.

Project Officers: George J. Galasso, Ph.D.; Hilton Levy, Ph.D.

Objectives: This contract has a two-fold purpose, the first being to establish an animal model of the disease which closely mimics a disease state in man in which potential new antivirals can be tested, and secondly to serve as a direct model in anticipation of clinical studies on the treatment of rabies where vaccine and interferon would be used simultaneously.

Major Findings: The effects of polyinosinic·polycytidylic (poly I·poly C) acid combined with exogenous interferon used as an enhancer for interferon production, the protective effects of exogenous interferon and interferon inducers when used in combination with anti-rabies vaccine, and similar experiments on the effect of varying doses of vaccine and poly I·poly C on the prevention of rabies in mice and rabbits are underway. The contractor will continue his work to determine optimal antiviral effects of the agents being studied to determine the limits of their effectiveness with respect to their time of administration after virus challenge and determine the minimal effective dose of the antiviral, the best sites of administration, the effect of enhancers of the antivirals, and the effects the antivirals may have on the outcome of the disease when used in combination with present day anti-rabies vaccine and antisera treatment. There have been some problems in obtaining results due to the unpredictable nature of the challenge rabies virus. This problem is inherent in the "street" variety, which could be overcome by using "fixed" virus; however, since we are interested in clinical application, the work should be done with the street virus. Methods of overcoming this problem have been achieved and the current contract year should prove fruitful.

Significance to NIAID Programs and Biomedical Research: Having firmly established these animal models, the program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the program a direct means of comparison in determining the relative merits of the various substances. This contract also serves as a preliminary study toward the adoption of a new method of treatment for rabies in man.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year, at which time it will be evaluated and a decision made on its continuance based on results obtained.

Date Contract Initiated: June 17, 1970

Current Annual Level: $42,819

UNIVERSITY OF UTAH - (NIH-72-2518)

Title: Antiviral Substances and Animal Systems

Contractor's Project Director: Lowell A. Glasgow, M.D.

Project Officers: George J. Galasso, Ph.D.; Hilton Levy, Ph.D.

Objectives: Provide an animal-model system for examining antivirals against virus diseases similar to those in man. Having provided baseline data concerning interferon and interferon inducers, this study will be available for comparison of new antivirals as they are developed. This contract includes viruses which are sensitive to interferon (EMC) and viruses which are relatively resistant (cytomegalovirus and Herpesvirus hominis) studied in the mouse.

Major Findings: The contractor has further refined his models so as to more accurately define the viral dosage and route of inoculation and thus obtain the most natural route of infection. He has further determined that polyinosinic·polycytidylic (poly I·poly C) confers significant protection against the lethality of EMC virus when given shortly after viral inoculation; however, the drug had to be administered prior to viremia. Preliminary studies with adenine arabinoside (Ara-A) and iododeoxyuridine (IDU) showed some effect in altering the pathogenesis of Herpes virus infections. The contractor has recently received several additional compounds from the Antiviral Substances Program/NIAID for evaluation. This group will also continue to study interferon induction and the role of enhancing compounds such as COAM.

Significance to NIAID Programs and Biomedical Research: Having firmly established these animal models, the program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the program a direct means of comparison in determining the relative merits of the various substances.

Proposed Course of Project: It is anticipated that this project will be continued through June 1974, when it will be re-evaluated, with continued support based on programmatic need.

Date Contract Initiated: June 30, 1970

Current Annual Level: $ 46,202

VANDERBILT UNIVERSITY (NIH 72-2507)

Title: Clinical evaluation of respiratory vaccines in children and infants.

Contractor's Project Director: David T. Karzon, M.D.

Project Officers: Robert M. Chanock, M.D. and June K. Dunnick, Ph. D.

Objectives: The contractor will conduct clinical evaluation of live virus vaccines (respiratory syncytial virus, parainfluenza viruses and adenoviruses) and of inactivated Mycoplasma pneumoniae vaccine. Currently, emphasis is on the evaluation of live respiratory syncytial virus vaccine in semi-closed populations of infants to ascertain infectivity, degree of virulence and production of antibody. The contractor is prepared to evaluate other viral vaccines as directed by the project officer.

Major Findings: During the first contract year, 2 trials of the RS-A2 ts-1 mutant viral vaccine were conducted in children over one year of age. A large amount of effort was put into establishing this center as a workable vaccine evaluation center. The initial problem of recruiting adequate numbers of volunteers has now been overcome due in large part to acceptance of the program by the community. This represents an extensive effort by the investigators to develop rapport with the community.

Significance to NIAID Program and Bio-Medical Research: If the IDB is to develop and evaluate new vaccines for the pediatric population, there is a definite need to do very careful phase I testing in carefully controlled populations. This facility fulfills this need. This contract provides a semi-closed facility wherein young children and infants can be kept under careful surveillance during the test period. They are also prepared to evaluate a variety of candidate viral vaccines as they become available through the IDB.

Proposed Course of Project: The contract is for a period of one year, through May 18, 1974, at which time it will be reviewed and considered for additional funding.

Date Contract Initiated: May 18, 1972

Current Annual Level: $99,641

VETERANS ADMINISTRATION HOSPITAL, WASHINGTON, D.C. (Interagency Agreement)

Title: Efficacy of hyperimmune anti-HB Ab gamma globulin for the prevention or modification of parenterally acquired viral B hepatitis.

Project Directors: H. J. Zimmerman, M.D. and L. B. Seeff, M.D.

Project Officers: Robert H. Purcell, M.D. and June K. Dunnick, Ph. D.

Objectives: The Veterans Administration Hospital, under the direction of Drs. Zimmerman and Seeff, will conduct a double-blind study administering intramuscularly (IM) either normal gamma globulin or high-titered anti-HB Ag gamma globulin to determine if these preparations lower the incidence of hepatitis B infection in certain "high risk populations" by preventing or modifying the disease.

Major Findings: Since this is a "double blind" study, the actual results will not be known until the code is broken. As of April 6, 1973, 75 persons have been admitted into the study to determine the effects of HB Ab gamma globulin administration in preventing hepatitis B after accidental "needle stick" exposure. In February 1973, a study to determine the efficacy of hepatitis B immune serum globulin for the prevention or modification of post-transfusion hepatitis was begun.

Significance to NIAID Program and Bio-Medical Research: The NIAID Collaborative Hepatitis B Program has as its goal, control of hepatitis B infection. Generally, control of hepatitis may be divided into two categories: 1) Environmental control, which includes elimination of the carrier state; obtaining high quality blood and blood products; and testing and elimination of HB Ag or hepatitis agents from blood and blood products 2) and control by immunization-either active or passive immunization.

The NIAID - VA Hospital Cooperative Hepatitis Study is designed to determine if hepatitis B infection can be controlled by passive immunization, and this study is thus an integral part of the overall NIAID Hepatitis Program.

Proposed Course of Project: 2-3 years to complete study

Date Contract Initiated: May 1972

Current Annual Level: $63,925

UNIVERSITY OF VIRGINIA (NIH 72-2508)

Title: Clinical Evaluation of Influenza Virus and *Mycoplasma pneumoniae* Vaccines

Contractor's Project Director: Jack Gwaltney, Jr. M.D.

Project Officers: Brian Murphy, M.D. and George J. Galasso, Ph. D.

Objectives: 1) To clinically evaluate attenuated influenza virus vaccines administered by the intranasal route in appropriate populations to show infectivity, degree of virulence and efficacy. 2) To clinically evaluate inactivated *M. pneumoniae* vaccine for safety and antigenicity in young adults. 3) To clinically evaluate inactivated influenza virus vaccines administered parenterally to show safety, antigenicity and efficacy.

Major Findings: A live attenuated influenza virus was administered intranasally to 71 adult volunteers. The vaccine strain is a recombinant of influenza A/Hong Kong/1968 ($H_3N_2$) virus and a temperature-sensitive (TS) lesion derived from a TS mutant of influenza A/Great Lakes/1965 ($H_2N_2$) virus. A single dose of 0.3 ml of material containing approximately $10^5$ $TCID_{50}$ of virus/ml was administered. Twenty-five additional men served as controls in the double-blind study. No serious illness resulted, and no symptoms in vaccinees were statistically significant when compared to controls. Only six men in the vaccine group showed a four-fold serum antibody response and five men shed virus after vaccination for a total infection rate of 9/71 (13%). The low serum antibody response may have been due to the boderline dosage used to infect the men. Virus transported to and from Goochland (elapsed time ≈ 4 1/2 hours) was one log lower than the starting material. Coarse drops (not a spray) were given and most men had pre-existing antibody. A repeat study using twice the initial dose in nine men with titers <20 is almost completed, none shed virus. Nasal antibody responses are being analyzed.

Significance to NIAID Program and Bio-Medical Research: Contractor provides unique populations which have been under surveillance, so that efficacy studies of influenza virus vaccines can be performed.

Proposed Course of Project: Renewal is probable

Date Contract Initiated: April 1, 1972

Current Annual Level: $65,000

WASHINGTON STATE UNIVERSITY - (NIH 70-2130)

Title: The Influence of Interferon and Interferon Inducers on the Pathogenesis of Feline Panleucopenia, Feline Respiratory Disease Complex, and on Canine Distemper in the Ferret

Contractor's Project Director: Jeffrey C. Duenwald, D.V.M.

Project Officers: George J. Galasso, Ph.D.; Hilton Levy, Ph.D.

Objectives: A series of animal models have been established for diseases which closely mimic viral diseases in man which need to be controlled. Having established baseline data on the effects of interferon and interferon inducers in prevention and/or treatment of these viral diseases, these models will serve as a facility for quickly and efficiently examining new antivirals as they are developed.

Major Findings: During the past year the contractor has examined the interferon-inducing capabilities of polyinosinic·polycytidylic acid (poly I·poly C), tilorone, statolon, Newcastle disease virus (NDV), and ultraviolet inactivated NDV. Their comparative merits have been studied, and under certain conditions the diseases have been partially ameliorated. Statolon produces the highest titer and the longest duration of response in the ferret. Poly I·poly C is a good inducer in both the ferret and the cat. The response elicited with poly I·poly C when combined with polybasic COAM, DEAE, or spermine is somewhat better than poly I·poly C alone. All three models have been perfected to simulate the natural disease condition, and the parameters employed to evaluate the disease course have been expanded.

The cat interferon assay system has been developed using a stable fetal cell line with good results. The ferret assay is subject to variability, and efforts are underway to find a suitable continuous cell line in preference to the primary cells currently being used.

During the past year the most efficient modes of inducing interferon were more clearly defined in both animal species and are currently being applied to the disease models.

Significance to NIAID Programs and Biomedical Research: Having firmly established baseline data on the effects of interferon and interferon inducers in these animal models, the program will be better able to directly test new antivirals as they are developed in the selected systems. This will afford the program with a direct means of comparison in determining the relative merits of the various substances and their potential for clinical use.

Proposed Course of Project: It is anticipated that this project will be continued through June 1974.

Date Contract Initiated: June 1, 1970

Current Annual Level: $55,093

MEDICAL COLLEGE OF WISCONSIN - (NIH-70-2125)

Title: Interferon Assay Systems

Contractor's Project Director: Sidney E. Grossberg, M.D.

Project Officers: George J. Galasso, Ph.D.; Samuel Baron, M.D.

Objectives: In order to support and expedite research in the field of interferon, an antiviral agent which may prove useful in man, a study was undertaken to develop a more rapid, quantitative and standardized assay. Toward this end this contract is examining a variety of cell lines and strains to determine the most sensitive. They will also examine nutritive and environmental culture conditions, methods of enhancing the interferon effect, optimal dose of viral challenge and enhancement of detection technique.

Major Findings: This productive contract has developed the following information: (a) identification of two human continuous cell lines reasonably sensitive to interferon; (b) development of a hemagglutinin yield-reduction assay using encephalomyocarditis virus; (c) comparative assays of several diploid and continuous human cell cultures for interferon sensitivity; (d) analysis of some of the environmental variables required for the interferon assay; (e) development of a neuraminidase yield-reduction assay which offers high promise for titration of human interferon as well as interferon from other species, and (f) extension of some of these studies to the assay of murine interferon. The contractor has been awarded an additional year to further define the neuraminidase assay. This assay has great potential in that it is accurate, reliable and can be easily adapted to various interferon systems. The current year is being spent at simplifying this assay so that it can be readily used in any laboratory.

Significance to NIAID Programs and Biomedical Research: The methods currently in use to assay interferon are tedious, time-consuming, and are not as reliable as would be desired. It is expected that this contract will yield information which would enable an investigator to do more assays in a shorter period of time with great reliability. This would encourage workers such as chemists to enter into this field as well as assisting the current investigators with their work.

Proposed Course of Project: The contract is in its terminal year. It is anticipated that the contractor will complete the assigned work task by June of 1974.

Date Contract Initiated: June 1, 1970

Current Annual Level: $39,771

MEDICAL COLLEGE OF WISCONSIN - (NIH-72-2524)

Title: Biological Inducers of Interferon

Contractor's Project Director: Sidney E. Grossberg, M.D.

Project Officers: George J. Galasso, Ph.D.; Samuel Baron, M.D.

Objectives: All attempts to date to yield a nontoxic interferon stimulator, whether biologically or artificially derived, have been unsuccessful. The effective dosage of interferon may be so high as to render the exogenous route prohibitive for many applications. There is therefore a need to develop an inducer which is both nontoxic and highly effective. An efficacious approach to this search may be an investigation of biological products. This is one of two contracts to determine which of the available biological inducers have clinical potential.

Major Findings: During the first year of the project, a number of biological products have been selected for evaluation of their relative merits as candidates for clinical application. While these materials were being collected and prepared, major emphasis was given to work on the soluble protein interferon inducer extracted from Escherichia coli 0111:B4:2. The crude E. coli cell extract consists of the 100,000 x g supernatant fluid obtained from differential centrifugation of sonically disrupted, heat-killed cells. This extract stimulated mice to produce high titers of circulating interferon and antiviral resistance to encephalomyocarditis virus. Various methods of extraction and treatment of the material are being investigated. Similar studies on the other selected inducers are currently being initiated.

Significance to NIAID Programs and Biomedical Research: Interferon holds great promise for clinical application. This can be achieved through exogenous administration or endogenous production through use of safe interferon inducers. This project is an attempt to obtain an inducer of biological origin which will prove effective and safe with the ultimate goal of use in man.

Proposed Course of Project: This contract will be continued through June 1976 with three years of incremental funding: the first phase for the acquisition, production and purification of selected biological inducers, the second phase for tissue culture and animal studies to determine the most effective inducers, and the final phase to determine the actual moiety of the inducer responsible for the interferon stimulation.

Date Contract Initiated: June 26, 1972

Current Annual Level: $72,119 (estimated)

UNIVERSITY OF WISCONSIN - (NIH-70-2132)

Title: Two Swine Viral Disease Systems for the Study of Interferon and Interferon Inducers

Contractor's Project Director: B. D. Easterday, D.V.M., Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: The establishment of an animal model system for diseases which closely mimic viral diseases in man which need to be controlled. These models are to be readily available for evaluation of new antivirals as they are developed.

Major Findings: This contract has well established the basic parameters of the piglet model using swine influenza and transmissible gastroenteritis viruses as the infecting agents. The infectious challenge dose and mode of inoculation which closely approximates the natural state has been determined. Baseline data using the interferon inducer polyinosinic·polycytidylic (poly I·poly C) acid and various enhancing compounds are being accumulated. Studies on the effectiveness of adenine arabinoside and iododeoxyuridine have been initiated although these drugs are effective against DNA viruses, and these models are RNA viruses. The ensuing data will be important in determining toxic effects. This contractor can also provide a model using a DNA virus (pseudorabies) when necessary.

A series of additional antivirals have been sent to this investigator for his evaluation.

Significance to NIAID Programs and Biomedical Research: Having firmly established these animal model systems, the program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the program a direct means of comparison in determining the relative merits of the various substances.

Proposed Course of Project: It is anticipated that this project will be continued through June 1976 under option to renew dependent on productivity and programmatic need.

Date Contract Initiated: June 18, 1970

Current Annual Level: $47,000

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SCIENTIFIC DIRECTOR'S SUMMARY
LABORATORY AND CLINICAL RESEARCH
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

July 1, 1972-June 30, 1973

The individual laboratory chiefs have summarized highlights of research from their laboratories, and these provide a solid record of accomplishment. To the extent that it provides a measure, the record of publications, presentations at national and international scientific meetings, of invited lectures, and of awards, marks the year as one of the most productive in the history of the intramural program.

Dr. Robert Chanock, Chief of the Laboratory of Infectious Diseases, received the Gorgas Medal from the Association of Military Surgeons of the U.S., was elected Vice President of the American Epidemiological Association, was invited to give the Annual Ricketts Lecture at the University of Chicago and was elected to the National Academy of Sciences. Dr. Wallace Rowe, Chief of the Laboratory of Viral Diseases, received a Rockefeller Public Service Award, gave an invited lecture in honor of Dr. Peyton Rous at the Rockefeller University, presented the fifth annual G. Burroughs Mider Lecture at the NIH and gave the G. H. A. Clowes Memorial Lecture at the American Association of Cancer Research in Atlantic City. Dr. Norman Salzman, Chief, Laboratory of Biology of Viruses, received a DHEW Superior Service Award. Dr. Charles Kirkpatrick of the Laboratory of Clinical Investigation was appointed Chairman of the International Committee of the American Academy of Allergy, and Dr. Alan Rosenthal is Chairman of the organizing committee for the 1974 meeting of the Leukocyte Culture Conference in the U.S. Dr. Kirkpatrick also served as a co-chairman in organizing and conduct of a workshop on transfer factor. Many others gave invited lectures or seminars, served on editorial boards, or otherwise participated in professional activities which reflected to the credit of both them and the NIAID.

The personal activities of the Scientific Director for the first ten months of the fiscal year were mostly influenced by the needs of the Collaborative Programs, by administrative problems related to further personnel reductions, and in a variety of other duties, including the Cholera Research Program. During the first six months of the fiscal year, considerable time and effort went into trying to recruit a new Associate Director for Laboratory and Clinical Research. This was after agreement with the Director, NIAID, and the Deputy Director for Science, NIH, that the position of the former Assistant Scientific Director for Laboratory and Clinical Research should be dropped and that the replacement for the former Assistant Scientific Director should take full and independent responsibility for the Laboratories. These efforts seemed crowned with success in September, 1972, when a prominent immunologist accepted. His university persuaded him to re-think the matter and recruiting began again. Another promising candidate had been found and undoubtedly would have accepted but in mid-year when news of the further personnel reductions reached the Institute, we decided to defer any further definitive action. On May 1, the

Scientific Director was relieved of his major responsibilities in the Collaborative Programs and, to his pleasure, returned to a nearly full time role in the Laboratory and Clinical Research Programs. He retains overall coordination responsibility for direct program and is overall Institute program officer for hepatitis and influenza programs.

There was no significant change in the staffing levels of the Laboratories during the year but personnel ceilings assigned the NIAID in December of 1972 required that we reduce staff by June 30, 1974, by a total of 42 positons overall or 35 in the Laboratory and Clinical Research Program. The NIH asked all Institutes to develop plans for making these reductions. Upon review and approval of the plan by the Deputy Director for Science, NIH, the Institute began implementation. The plan calls for disestablishment of the Laboratory of Microbiology as presently constituted. A small Laboratory of Streptococcal Diseases was established to continue the work of Dr. Roger Cole and his immediate associates. The mycology genetics program was combined with the medical mycology program of the Laboratory of Clinical Investigation. The mycoplasma section was transferred to the Laboratory of Infectious Diseases and consolidated with mycoplasma research in that Laboratory. The balance of research in the Laboratory of Microbiology is to be phased out.

The Board of Scientific Counselors met twice during the year. In November, 1972, it reviewed the research of the Laboratory of Microbiology, singling out those activities slated for continuation as described above as of high scientific quality. The other areas were considered to have less future promise. The Board again met in April of 1973 to review all Laboratory and Clinical Research programs in connection with Institute plans for effecting personnel reductions. Several alternatives were discussed. The Board was unanimous in its support of the NIAID position that personnel reductions should be achieved by elimination of weaker research programs rather than by attrition. They went further, recommending that sufficient program and associated staff be eliminated to enable creation of positions to repair damage done in past reductions taken by attrition.

The Cholera Research Laboratory (CRL) in Dacca, Bangladesh, continued to operate in what must be regarded as a holding operation. It was funded by the Agency for International Development (AID) through a grant to the International Rescue Committee. This enabled the indigenous staff to be kept together and the hospital facilities to be operated for the purpose of training local physicians and paramedical people in the treatment of diarrheal diseases. Dr. W. Kendrick Hare continued on as Director. Other American staff in residence were Mrs. (Dr.) Hare, Mr. Frank Eddy, Mr. Mark Tucker, and Miss Lillian Wall. Dr. David Nalin of the Johns Hopkins International Center for Medical Research was also in residence and was the principal focus of research. Dr. Stephen Richardson, Chairman, Department of Microbiology at Bowman-Grey, spent a six-months' sabbatical at the Laboratory in studies on cholera and *E. coli* toxins.

Negotiations between AID and the Covernment of Bangladesh and between the NIH and AID continued throughout the year towards reaching a new agreement for the operation of CRL. Only a few minor details prevented this being formalized

during FY 1973 and agreement is expected early in FY 1974.

The major NIH interest in perpetuation of CRL has been to provide a field site for the testing of a cholera toxoid. Wyeth Laboratories, developing the toxoid under contract, solved most of the difficulties which beset earlier efforts. When it became apparent that a stable toxoid could not be made with formalin, they substituted gluteraldehyde. While the product was not as antigenic as had been the earlier, unstable formalin toxoid, this was correctable to a large degree by the ability to use adjuvants. Doses of up to 100 mcg. were given to volunteers with adjuvant and were acceptable from the standpoint of reactions. Plans were perfected for beginning studies in Bangladesh in July of 1973 directed towards a full field trial for efficacy in the summer of 1974.

The former Middle America Research Unit was operated under contract with the Gorgas Memorial Institute throughout the year. Six professional staff members remained as part of the Scientific Director's office to complete their work. These were Drs. Johnson, Webb, Peters, Dietz, Reeves, and Chaniotis. Dr. Tesh left in December to join the Pacific Research Section of the Laboratory of Parasitic Diseases. Dr. Chaniotis left in May for a one-year visiting scientist appointment at the Rocky Mountain Laboratory. Drs. Peters, Dietz, and Reeves left in June, 1973. The full report of their work is incorporated in the progress report of the Gorgas Memorial Institute in contract files and will be reflected in the annual report of the Institute to Congress. A few highlights follow.

Tacaribe Virus Vaccine

A high passage strain of Machupo virus was purified and shown to infect but not kill guinea pigs and marmosets. These animals were completely protected against challenge. The attenuated strain was adapted to growth in human diploid cells. Markers for attenuation were identified.

Venezuelan Equine Encephalitis (VEE)

The hypothesis of relation of antigenic variation and virulence of various strains was further substantiated by experimental studies in horses. A strong correlation was found between virulence and two *in vitro* markers, virus plaque diameter and pH optimum for hemagglutination. Multiplication of virulent and avirulent viruses was primarily in lymphatic tissues with virulent viruses growing to much higher titers. Both caused death when inoculated intrathecally but avirulent virus did not escape the brain whereas virulent virus did.

Viruses of Phlebotomine Sandflies

Six new members of Phlebotomus fever arbovirus group were characterized. One was shown to produce infection in man.

Hepatitis

Studies were completed on 600 hepatitis patients hospitalized in Panama. Hepatitis B antigen was detected in 22 of 32 post-transfusion cases, in six of

seven following use of parenteral narcotics, and five of nine with an occupational exposure to blood. It was detected in 20% of 537 sporadic cases of hepatitis. Only three of 166 children studied had antigenemia. Prospective studies of family contacts were carried out. Only 3 of 60 adult contacts and 4 of 41 children became infected. Only 2 of 10 sexual partners became infected.

Studies on carriers of hepatitis B antigen in various population groups were completed. The incidence varied was as follows: inhabitants of 22 towns in interior of Panama (17/1938 = 0.9%), of Panama City (2/642 = 0.3%), a West Indian black community in the Canal Zone (2/166 = 1.2%), Cuna Indians (0/538) and Choco Indians (2/298 = 5%). Guaymi Indians living on the Pacific (21/787 = 3.0%) or Atlantic (20/241=8.3%) watersheds of Panama had a significantly higher prevalence of antigenemia. Antibody studies on a random selection of sera from these subjects indicated that antibody was present in 8% of sera from Guaymi Indians aged 19 or less and in 30 to 50% of adults. Choco Indians had a greater prevelance of antibody. No explanation could be found for the lesser rate of antigenemia in Chocos as compared to Guaymis in view of comparable or higher levels of antibody indicating exposure in the former.

From 15 to 40% of various new world species of monkeys had anti-HB Ag antibody. Included were seven species of monkeys wild caught in Panama and adjacent areas of Columbia.

LABORATORY OF CLINICAL INVESTIGATION

Summary . . . . . 1

10(c) - Office of the Chief . . . . . 9

11(c) - Clinical Care Program in Support of Research Projects . . . . . 11

12(c) - Mechanisms of Fever and Host Responses . . . . . 12

14(c) - Medical Virology - Investigations of Host Response in Murine Coxsackie $B_3$ Myocarditis (Revised Title) . . . . . 20

18(c) - Immunology and Chemotherapy of Systemic Mycoses (Revised Title) . . 23

23(c) - Observations on Secretory Immunoglobulins (Primarily Canine, but Includes Rabbits and Guinea Pigs also) (Revised Title) . . . . . 31

30(c) - Studies on the Interaction of Antibody and Complement in the Production of Immune Damage . . . . . 34

33(c) - Pathogenesis of Delayed Hypersensitivity . . . . . 39

36(c) - The Structural Correlates of Antigen and Mitogen Interactions with Immunocompetent Cells . . . . . 56

40(c) - Clinical Studies on Patients with Known or Suspected Parasitic Diseases . . . . . 60

44(c) - The Pathophysiology of Autoimmune Hemolytic Anemia . . . . . 63

45(c) - Ultrastructural Correlates of the Cellular Events in Host Defense Mechanisms in Man and Experimental Animals . . . . . 66

46(c) - Immunobiologic and Ultrastructural Characterization of the Mechanism of Antigen Recognition (Revised Title). . . . . 69

47(c) - Clinical Studies of Complement Abnormalities of Man . . . . . 74

48(c) - Antibody Responses in the Respiratory Tract of Rabbits Following Parenteral Immunization with Pseudomonas Aerginosa Vaccine and Enhancement of Bacterial Phagocytosis and Killing by Respiratory Macrophages with Immune Opsonic Antibodies . . . . . 77

49(c) - Laboratory Studies on Viruses of Entamoeba hystolytica and their Possible Significance in Human Amebiasis . . . . . 81

50(c) - Mechanisms in Host Defense . . . . . 83

51(c) - Human Responses to Salmonella typhosa Vi Antigen (Clinical Research Project #73-I-01) . . . . . 87

52(c) - Antibiotic Kinetics in Respiratory Secretions . . . . . 90

53(c) - The Etiology and Clinical Characteristics of Acute Infectious Nonbacterial Gastroenteritis . . . . . 93

54(c) - Regulation of the Immune Response in Man and Experimental Animals . . . . . 96

55(c) - Granulocyte Physiology . . . . . 99

56(c) - The Biochemical Pathways of Mediator Release and Mechanism of Tissue Injury in Allergic Diseases . . . . . 103

Summary of Program
Laboratory of Clinical Investigation
July 1, 1972 - June 30, 1973

Sheldon M. Wolff, M. D., Chief of Laboratory
and Clinical Director, NIAID

## Introduction

Despite the fiscal and personnel constraints of the past year, the Laboratory of Clinical Investigation has continued to mature, achieve stability and to be productive. In fact, in terms of output of publications, the past year has been the most productive in the history of the Laboratory. Significant contributions have been made, and it is a source of considerable pride to us that members of the Laboratory are receiving increasing recognition by the scientific community.

The losses of Drs. Harry R. Kimball and Julius A. Kasel last year were major ones. Dr. Kimball's studies dealing with lysosomes had to be abolished, and this, unfortunately, has left a gap in the overall program. Fortunately, we shall be able to continue our virology program despite Dr. Kasel's departure. We are looking forward to Dr. Raphael Dolin's return to the National Institute of Allergy and Infectious Diseases from Boston. His laboratories have been remodeled to meet his needs, and we anticipate a rapid activation of his program dealing with the pathogenesis of viral diseases.

The return of Drs. David C. Dale and Anthony S. Fauci and the addition of Dr. Allen P. Kaplan have markedly increased not only the size but the capability of the Laboratory. Drs. Dale and Fauci are already making significant progress in their research, and they have added enormous clinical strength to our infectious disease program. Dr. Kaplan's program in allergy has begun, and his laboratory is now fully operational. The clinical program is just beginning, and we hope that this aspect will soon begin to grow.

In spite of fears concerning the availability of good applicants for our associate program now that the draft has ended, we have received a large number of applications from highly qualified and capable young men. Thus, we anticipate the continuation of a steady input of bright young people into our Laboratory. We continue to supply the Naval Hospital with consultative support in infectious diseases, and this effort has proven worthwhile and rewarding for both our staff and our colleagues at the Naval Hospital.

During the coming year a formal clinical clerkship in infectious diseases will begin. During the past year we have had such clerks but only in a sporadic fashion. Of interest is the increasing numbers of scientists and clinicians who are spending periods of time in the LCI. A large number of residents from the U. S. Naval Hospital, Georgetown and George Washington University have spent time with us this past year. Scientists from Yugoslavia, England, France and South Africa have also worked in the LCI.

Our clinical programs are active and strong. Both wards under our auspices have been busier this past year than at any time since 1966. We have received tremendous support from our nursing service, despite very difficult staffing problems.

During the past year members of the LCI have been invited to participate in a large number of symposia, courses, etc. In addition, papers were presented at a wide variety of meetings. Dr. Rosenthal is in charge of the Leukocyte Culture Conference to be held in the U.S.A. in 1974. Dr. Kirkpatrick is the Chairman of the International Committee of the American Academy of Allergy, and he was Co-Chairman of a Workshop on Transfer Factor. Dr. Frank is on the faculty of the immunology course given by the American Association of Immunologists. Many other members of the staff of the LCI are involved in such endeavors, but space does not permit a complete listing.

Although the LCI is strong and productive, we continue to face major difficulties. Last year we called attention to our space problems. No relief has been given, and we will experience increasing difficulties if we cannot solve this problem. Furthermore, the restrictions on personnel have severely limited the natural growth of some of our programs. We sorely need more technical and support staff. Many of our investigators have only a single technician, and we are very much dependent upon part-time, "stay-in-school," and volunteer high school students to perform needed tasks. Such dependence is obviously not a permanent or satisfactory solution to our problem. Future difficulties will also surely arise if we do not have the support or capacity to keep some of our more talented young associates here for longer periods.

## Research Accomplishments

The Laboratory continues to place major emphasis on studies relating to clinical immunology, virology, mycology and host defense mechanisms. The role and function of blood cells as major determinants of resistance to infections are extensively investigated.

### CHEMOTAXIS

A simple, objective, and rapid radioassay of granulocyte chemotaxis employing $^{51}Cr$-labeled leukocytes has been perfected. This radioassay has been used to study the generation of chemotactic factors and the mechanism of leukocyte response to chemotactic stimuli. Kinetic analysis of the generation of complement-related chemotactic factors by the classical and alternate complement pathways provided a simple means to distinguish these pathways; such distinction is important clinically since normal chemotactic activity may be present despite complete absence or inhibition of one pathway. Noncomplement-related chemotactic factors were generated by activation of Hageman factor, demonstrating an interesting association between coagulation and inflammation. A new chemotactic factor was discovered in dialyzable transfer factor that through partial purification was inseparable from the transfer factor moiety; this chemotactic property of transfer factor may be an important "co-factor" for proper interpretation of immunologically-mediated inflammatory responses. Divalent cations were found to be important regulators of leukocyte chemotaxis. Both calcium and magnesium were demonstrated to be required for and synergistically support

optimal human granulocyte chemotaxis. Exposure of granulocytes to purified chemotactic stimuli resulted in a rapid calcium efflux which, on the basis of additional studies, is an important prerequisite for leukocyte chemotaxis. (Gallin, Frank, Kirkpatrick, Kaplan).

## CELLULAR IMMUNITY IN CRYPTOCOCCOSIS

A variety of studies suggested that opsonins and chemotactic factors generated during the C3-C9 sequence of complement activation play an important role in resistance to infection with Cryptococcus neoformans. Activation proceeded primarily through the "alternate" pathway, though the antibody-mediated, "classical" pathway was also necessary for optimum kinetics. The lack of this defense mechanism in cerebrospinal fluid was demonstrated by the lack of C3 on the surface of cryptococci found in patients' cerebrospinal fluids, as well as by the inability of CSF to opsonize cryptococci. This finding may help explain: 1) why man cannot eradicate cryptococcosis once infection is established in the central nervous system, in contrast to lung; and 2) why the inflammatory response to cryptococci is so sparse in infected brain and cerebrospinal fluid, in contrast to lung. (Diamond, May, Bennett, Kane, Frank).

Two in vitro correlates of delayed hypersensitivity have shown that cryptococcosis patients may have defective cellular responses to cryptococcal antigens. The tests employed were lymphocyte transformation and migration inhibition of peripheral blood leukocytes. (Schimpff, Bennett).

## KININS

Studies of the mechanism of generation of bradykinin in human plasma and the interrelationship of this pathway with the coagulation, fibrinolytic, and complement pathways are being pursued. During the past three years, three Hageman factor dependent pathways have been characterized by isolating the substrates of activated Hageman factor, pre-PTA, prekallikrein and plasminogen proactivator, which are required for the intrinsic coagulation, kinin-generating, and fibrinolytic pathways, respectively. The role of plasma inhibitors of the active enzymes has been studied and two chemotactic factors have been identified. These interrelated enzymatic pathways comprise one aspect of the inflammatory response which is being evaluated in patients with various types of chronic urticaria and chronic angioedema. The primary focus is upon idiopathic forms in which the vasoactive mediators are unknown. Other types of chronic urticaria and angioedema being studied include hereditary angioedema, cold urticaria, cholinergic urticaria, and urticarial erythema multiforme. In the hereditary form of angioedema, the pathogenic peptides appear to be bradykinin and a kinin derived from the complement pathway. The mechanism by which the latter peptide (C-kinin) is released and the manner in which it interacts with the bradykinin generating pathway are being investigated. In order to properly assess the kinin-generating pathway in clinical states, radioimmunoassays for the determination of Hageman factor, prekallikrein, kininogen and bradykinin are being developed. (Kaplan).

## FLETCHER FACTOR

"Fletcher factor" deficient plasma possesses a diminished rate of activation of the intrinsic coagulation pathway in the absence of a deficiency of any

known coagulation factor. Contact activation of Hageman factor in this plasma has been shown to yield no bradykinin because it is deficient in prekallikrein. A defect has been described in kaolin-activated fibrinolytic activity and in kaolin-activated chemotactic activity in this plasma. The coagulation, fibrinolytic, and chemotactic defects can be corrected by prolonged incubation with kaolin or by reconstitution with either prekallikrein or activated Hageman Factor. The kinin-generating defect is corrected only by reconstitution with prekallikrein. Furthermore, the product of Hageman factor activation of prekallikrein, i.e., kallikrein, activates inactivated Hageman factor. Thus, a feedback mechanism exists in which kallikrein activates Hageman factor without which the three Hageman factor dependent pathways proceed at a markedly diminished rate. The results indicate that the activation of Hageman factor which leads to coagulation, fibrinolysis,and kinin generation requires not only contact activation to initiate the process but also enzymatic activation of Hageman factor in the fluid phase. (Kaplan).

IMMUNOSUPPRESSION

Studies have been aimed at delineating the precise mechanisms whereby immunosuppressive agents such as corticosteroids and cytotoxic drugs affect the immune response in man and in experimental animals. Specifically, the project is aimed at looking at the in vivo and in vitro function of subpopulations of lymphocytes as well as the monocyte and how these functions are altered in diseases in which inflammatory or hypersensitivity phenomena play a significant role such as Wegener's granulomatosis, vasculitic, and granulomatous disease of various etiologies. The mechanisms whereby immunosuppressive agents alter the numbers, distributions, and function of these cells will be the focus of study. This will naturally lead into studies to determine the precise role of these populations of cells in host defense against infections and how this role is altered by manipulation of the immune response. (Fauci).

GRANULOCYTE PHYSIOLOGY

Studies of cyclic neutropenia in man have shown that the human disease occurs because of periodic failure of marrow cell formation. These findings closely parallel our studies of the similar disorder in grey collie dogs. Studies of 25 patients with chronic idiopathic neutropenia have indicated that as few as 200 to 500 circulating blood neutrophils may be compatible with infrequent infections. Chronic alternate-day adrenocorticosteroid therapy has elevated the blood neutrophil count in several of these patients and proved to be a useful treatment for two patients with severe neutropenia and recurrent infections. Effective immunosuppression therapy with cyclophosphamide in Wegener's granulomatosis does not require neutropenia or suppression of neutrophil inflammatory responses and by careful attention to leukocyte counts infectious complications are very infrequent with this single drug immunosuppressive therapy. (Dale, Guerry).

WHITE CELL FUNCTION

The contribution of peroxidase to neutrophil bactericidal activity occurs only during the first thirty minutes following phagocytosis. (Bujak).

Peritoneal exudate leukocytes from the beige variant of C57 black mice phagocytize but fail to kill S. aureus and group D streptococci as well as C57 black controls. Since these cells also have abnormal chemotaxis, the beige mice share the abnormalities of leukocyte function known to occur in the Chediak-Higashi syndrome of man. (Bujak, Gallin, Wolff).

Serum from uremic rabbits is less effective than pre-uremic serum in opsonizing S. aureus. This decreased activity does not correlate the alterations in blood urea nitrogen, creatinine, electrolytes, and glucose that occur with uremia and is still present in serum heated 56°C for 30 minutes. (Bujak).

## NON-SPECIFIC RESISTANCE

Fever was shown to alter the metabolism of BSP, antipyrine and iron in man. The rate of metabolism of BSP and antipyrine decreased significantly with fever. The serum iron concentration also decreased significantly during a febrile states. (Elin, Wolff).

The Limulus amoebocyte lysate test is not specific for endotoxin. Synthetic polynucleotides and activators of the mammalian coagulation system will effect a positive Limulus test. (Elin, Wolff).

Chediak-Higashi mice have a significant increase in mortality rate when challenged with Candida albicans, Staphylococcus aureus and Streptococcus pneumoniae compared with control mice, which is not due to hypogammaglobulinemia. (Elin, Wolff).

## VIROLOGY

The host response of volunteers experimentally infected with the viral agents of acute infectious nonbacterial gastroenteritis was examined. Definite but reversible pathophysiologic lesions, including jejunal epithelial changes and intestinal enzyme deficiencies, were noted in intestinal biopsies during acute illness. Biopsies taken two weeks after illness had returned to normal. In vitro IgA synthesis patterns in biopsies from ill and well volunteers were consistent with a possible role of local immunity to these viral agents. (Dolin).

## PULMONARY HOST DEFENSES

The general area of pulmonary host defenses and nosocomial infections is being investigated, using Pseudomonas pneumonia as the prototype infection because of its frequent occurrence in immunosuppressed patients who are most susceptible to opportunistic micro-organisms. Initial work has been done in rabbits to explore ways of manipulating antibody production in the respiratory tract and to assess the effectiveness of these antibodies in promoting phagocytosis and killing by alveolar macrophages. IgG was found to be the principal opsonin in the lung; maximal amounts of IgG opsonic antibody could be produced with a combination of intramuscular and intranasal (local secretory IgG produced) immunization. IgA antibody was induced with intranasal vaccination but was judged to have less opsonic activity. Competent phagocytic cells are essential for the pulmonary defenses. (Reynolds).

ANTIBIOTIC CONCENTRATION IN THE LUNG

Frequently antibiotic therapy is ineffective in severe pulmonary infections such as Pseudomonas pneumonia becuase adequate concentrations of the drugs are not obtained in the lung or respiratory secretions. Relatively little information is available about the penetration of antibiotics into the lung secretions or about the clearance from them. Studies in a dog model with gentamicin, tobramycin, and carbenicillin have shown that maximal concentrations of these drugs in bronchial secretions range from 15-30% of maximal serum values and that 30-50% of Pseudomonas aeruginosa isolates may not be adequately inhibited by these levels of antibiotics. However, the clearance of antibiotics from the bronchial secretions is relatively prolonged compared with serum (T-1/2 in bronchial secretion is two to three times longer), suggesting that the effective concentrations in the bronchial secretions may be longer, if adequate levels could be achieved. (Reynolds, Pennington).

SECRETORY IMMUNOGLOBULINS

The ultrastructure of canine secretory component and J-chain are being investigated now that they have been isolated and purified from secretory IgA; the combination of these chains with 7S IgA monomer may elucidate the assembly of the secretory IgA molecule. (Reynolds).

PEYER'S PATCH LYMPHOCYTES

While investigating the origin of precursor cells that differentiate into immunoglobulin secreting plasma lymphocyte cells in the gastrointestinal tract, the interactions of orally administered antigens and processing cells (T-lymphocytes) in gut-associated lymphoid tissue (Peyer's patches) were studied in adult guinea pigs. A detailed identification of the lymphoid cell populations in Peyer's patches was made. (Reynolds, Levin).

CELL-MEDIATED IMMUNITY

The studies of chronic candidiasis as a model of defective cell-mediated immunity have been extended and now include 21 patients. The prediction that clinical and immunological characterizations would serve to identify various classes of patients has been borne out. Furthermore, these studies have facilitated selection of treatment schedules. One mode of therapy, restoration of cell-mediated immunity with transfer factor, is apparently beneficial. However, the mechanism of action of transfer factor and the importance of using donors with positive Candida skin tests has not been established. (Kirkpatrick).

Characterization of the physico-chemical properties of transfer factor is underway. Thus far, a unique chemotactic substances in transfer factor has been identified, and some preliminary assays of its composition have been completed. (Kirkpatrick, Gallin).

LYMPHOCYTES

A major goal has been the characterization and separation of the antigen recognition unit in cell-mediated immunity. Initial studies separated thymus-derived (T) from bone marrow-derived (B) lymphocytes in the guinea pig and demonstrated

that there exists compartmentalization of distinct subsets of T cells. Those lymphocytes which enter non-specific inflammatory sites exhibit heightened antigen reactivity for those antigens to which the animal has been most recently immunized. Thus, exudate lymphocytes have greater antigen reactivity than lymph node lymphocytes from the same animal. Ultrastructural features of these cells showed that the exudate lymphocyte has a high frequency of uropods which is in support of the thesis that these are distinct subsets.

It has been further shown that the antigen recognition unit in cellular immunity involves a macrophage. This requirement is obligatory, and it has been demonstrated that recognition of soluble antigen requires an initial metabolic-dependent uptake of antigen by the macrophage antecedent to immunospecific T-cell recognition. Ultrastructural studies in progress demonstrate that internalization of antigen is required and surface membrane antigen is not necessary as previously believed. (Rosenthal, Lipsky).

HYDROCORTISONE AND LYMPHOCYTES

Hydrocortisone blocks the action of a product of cell-mediated immunity known as macrophage inhibitory factor (MIF). Furthermore, this blockage is not due to the inability of lymphocytes to recognize an antigen or produce MIF. Instead, steroids block the effect of MIF on the macrophages themselves, either by interference with a cell surface receptor or by inducing a metabolic event which is antagonistic to the decreased migration of macrophages. These data identify, for the first time, a specific *in vitro* site of hydrocortisone action on cell-mediated immunity and may provide insight into the way by which steroids alter host defense to external challenge. In addition, these studies suggest a mechanism by which this drug may alter cell-mediated immunity against self, as in arthritis, and may explain the usefulness of steroids in the treatment of such conditions. (Rosenthal, Balow).

COMPLEMENT PATHWAYS

A detailed series of studies have established the role of the various pathways of complement activation in biological phenomena mediated by complement. The alternate pathway has been shown to efficiently mediate Arthus reactions. Many events of inflammation are also mediated by the alternate pathway, although in the case of some, such as chemotactic factor generation from complement and phagocytosis of organisms and lysis of bacteria, the alternate pathway proceeds after a kinetic delay. The function of immune surveillance with the damage of foreign cells and tissues mediated by antibody is primarily mediated through activation of the classical pathway. A new pathway has been identified whereby specific antibody and C1, a classical pathway component, can activate the alternate pathway thereby inducing cell damage. (Frank, May, Kane).

COMPLEMENT IN DISEASE

Clinical studies of complement component function have disclosed the first case of C6 deficiency in man and have allowed for genetic analysis of its inheritance. C6 was shown to follow a simple Mendelian inheritance with heterozygous-deficient individuals with half normal levels and homozygous-deficient individuals with none. Studies of the complement component C4 in cerebrospinal fluid (CSF) have

shown that individuals with central nervous system involvement with lupus erythematosus have low levels of CSF C4 during the acute attack; however, the range of normal is wide and one individual titer does not provide sufficient information to establish this diagnosis. (Frank, Gerwin).

AUTOIMMUNE HEMOLYTIC ANEMIA

Studies of the pathophysiology of autoimmune hemolytic anemia in a guinea pig model have continued to provide information about the role of antibody and complement in the immune clearance and destruction of erythrocytes. Earlier studies had established the importance of immunoglobulin class on the extent and pattern of clearance of sensitized cells from the circulation. They had also demonstrated that the classical complement pathway rather than the alternate pathway was responsible for the observed clearance. New studies have demonstrated that corticosteroids can inhibit clearance of the sensitized cells, that four to five days of therapy are required before this effect is manifest and that steroids modify the clearance rates of cells with low levels of sensitization more than cells with higher levels of sensitization. Splenectomy affects clearance of IgG-sensitized cells alone but has a very profound effect on the clearance of these cells, especially at low levels of sensitization. (Frank, Atkinson).

ENTAMOEBA HYSTOLYTICA

Recognition of viruses of Entamoeba hystolytica latent in amebic cell lines maintained axenically for long periods of time in the laboratory prompts an investigation of their role, if any, in the pathogenesis of human amebiasis. An additional E. hystolytica isolate freshly obtained from a patient with amebic liver abscess and empyema was found to contain virus when tested after the fifth passage in culture with bacterial associates. Cell lines which had been maintained in axenic culture for long periods continued to produce virus when reassociated with bacteria and remained susceptible to exogenous virus. The study of isolates from asymptomatic carriers was hampered by difficulty in obtaining such isolates from patients who could be well studied clinically. Bacteria-associated E. hystolytica isolates from two symptomless individuals were examined, and no virus could be demonstrated with the techniques employed. (Franck).

PARASITOLOGY

Most of the patients admitted to the Clinical Center in connection with this project over the past year were either Peace Corps personnel or African students with schistosomiasis. Amebiasis, toxoplasmosis, giardiasis, and leishmaniasis constitute the other major diagnostic possibilities in patients seen in the Out-Patient Department or in-house. A surprisingly high failure of cure rate was found in a small series of schistosomiasis cases treated with Astiban: two of four cases. The studies on circulating schistosome antigen are being reported separately (by Dr. Nash) as well as the complement alterations in malaria patients. (Neva, Nash, Franck).

Serial No. NIAID-10(c)
1. LCI
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Office of the Chief

Previous Serial Number: SAME

Principal Investigator: Dr. Sheldon M. Wolff

Other Investigators: NONE

Cooperating Units: NONE

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 1-3/12 | | | 1-3/12 |
| Professional: | 3/12 | | | 3/12 |
| Other: | 1 | | | 1 |

Project Description:

Objectives:

To develop, direct and coordinate the program of the Laboratory, as defined in the individual research projects; where necessary, to redirect individual projects to meet current needs and advances in the field; to align clinical projects with patient availability; to provide an unexcelled standard of patient care for patients utilized in research.

Methods Employed:

Organize available staff and recruit qualified personnel, both professional and sub-professional, to develop the program and carry out its aims; maintain close liaison with area medical societies, institutions and individuals for referral of patients whose diagnoses fall within the active or proposed disease research categories; the highest standards of patient care are maintained by selection of qualified physicians; the development of necessary policies and continued close and direct supervision of patient care activities; professional consultant services to other institutes in the area of infectious diseases; continued guidance of research projects undertaken by younger investigators; maintenance of staff morale.

Proposed Course: To continue

Publications:

1. Allen, P. Z. and Johnson, J. S.: Studies on equine immunoglobulins. III. Antigenic interrelationships among horse and dog γG globulins. Comp. Biochem. Physiol. 41B: 371-383, 1972.

2. Clark, R. A., Hamory, B. H., Ford, G. H., and Kimball, H. R.: Chemotaxis in acute renal failure. J. Infect. Dis. 126: 460-463, 1972.

3. Clark, R. A., Kimball, H. R., and Padgett, G. A.: Granulocyte chemotaxis in the Chediak-Higashi syndrome of mink. Blood 39: 644-649, 1972.

4. Kaltreider, H. B. and Johnson, J. S.: Porcine immunoglobulins. I. Identification of classes and preparation of specific antisera. J. Immunol. 109: 992-998, 1972.

5. Kaltreider, H. B. and Johnson, J. S.: Porcine immunoglobulins. II. Antibody response of adult swine to bovine γ globulin. J. Immunol. 109: 999-1002, 1972.

6. Northrup, R. S. and Chisari, F. V.: Response of monkeys to immunization with cholera toxoid, toxin and vaccine: reversion of cholera toxoid. J. Infect. Dis. 125: 471-479, 1972.

7. Northrup, R. S., Doyle, M. A., and Feeley, J. C.: In vitro susceptibility of El Tor and classical Vibrio cholerae strains to trimethoprim and sulphamethoxazole. Antimicrob. Agents Chemother. 1: 310-314, 1972.

8. Root, R. K. and Stossel, T. P.: Functional comparison of Chediak-Higashi syndrome and chronic granulomatous disease leukocytes. In Bergsma, D. (Ed.): Birth Defects: Original Article Series. The Fourth Conference on the Clinical Delineation of Birth Defects. Part XIV: Blood. Baltimore, The Williams and Wilkins Co., Vol. 8, 1972, pp. 99-104.

9. Simon, H. B. and Sheagren, J. N.: Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. Cell. Immun. 4: 163-174, 1972.

10. Simon, H. B. and Sheagren, J. N.: Migration inhibitory factor and macrophage bactericidal function. Infect. Immun. 6: 101-103, 1972.

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12. Smith, J. W. and Utz, J. P.: Progressive disseminated histoplasmosis. A prospective study of 26 patients. Ann. Intern. Med. 76: 557-565, 1972.

Serial No. NIAID-11(c)
1. LCI
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Clinical Care Program in Support of Research Projects

Previous Serial Number: SAME

Principal Investigators: Dr. Sheldon M. Wolff
Dr. John P. Atkinson

Other Investigators: Dr. James E. Balow
Dr. Michael J. Chusid
Dr. Jerrold J. Ellner
Dr. Dirk K. Greineder
Dr. Louis W. Heck
Dr. Douglas L. Hurley
Dr. Eric A. Ottesen
Dr. David A. Ascarelli
Dr. Anthony P. Belmont
Dr, Joseph J. Chanda
Dr. Michael L. Cowan
Dr. Bernard David
Dr. Fred A. Gill
Dr. Gary D. Graham
Dr. Sands K. Irani
Dr. Joseph A. Kaufman
Dr. Thomas E. Walsh
Dr. Gilbert White

Cooperating Units: NONE

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 4-4/12 | | 1-11/12 | 6-3/12 |
| Professional: | 4-4/12 | | 1-11/12 | 6-3/12 |
| Other: | -- | | -- | -- |

Project Description:

Responsibility for care of patients in the various research areas.

Proposed Course: To continue.

Publications: NONE

Serial No. NIAID-12(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mechanisms of Fever and Host Responses

Previous Serial Number: SAME

Principal Investigator: Dr. Sheldon M. Wolff

Other Investigators:
Dr. David C. Dale
Dr. Anthony S. Fauci
Dr. Ronald J. Elin
Dr. Joseph S. Bujak
Dr. DuPont Guerry, IV
Dr. Charles A. Dinarello
Dr. John I. Gallin
Dr. Alan S. Rosenthal
Dr. Allen P. Kaplan
Dr. Michael M. Frank
Dr. Charles H. Kirkpatrick
Mr. Stanley B. Ward
Mr. Joseph B. Edelin

Cooperating Units:
Dr. David W. Alling, OSD, NIAID, NIH (no project no.)
Dr. Warner Barth, Washington Hospital Center, Washington, D. C.
Dr. Robert A. Clark, Harborview Hospital, Department of Medicine, Seattle, Washington
Dr. George G. Glenner, LEP, NIAMDD, NIH (no project no.)
Dr. Harvey R. Gralnick, CP, CC, NIH (no project no.)
Dr. Robert I. Henkin, ETB, NHLI, NIH (no project no.)
Dr. Herbert D. Hochstein, LCA, BB, NIH (no project no.)
Dr. Robert Horn, Vanderbilt University School of Medicine, Department of Pathology, Nashville, Tennessee
Dr. Ralph E. Johnson, DCBD, NCI, NIH (no project no.)
Dr. Arthur S. Levine, DCT, NCI, NIH (Project no. NCI-6830)
Dr. Seymour Perry, DCT, NCI, NIH (no project no.)
Dr. Robert E. Scott, University of Minnesota School of Medicine, Department of Pathology, Minneapolis, Minnesota
Dr. Elliot Vesell, M. S. Hershey Medical Center, Department of Pharmacology, Hershey, Pennsylvania

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 5-4/12 | | | 5-4/12 |
| Professional: | 3-4/12 | | | 3-4/12 |
| Other: | 2 | | | 2 |

Project Description:

Selected patients are being studied on the wards and in the laboratory. The patients can be divided into the following groups: patients with a) recurrent fever; b) Chediak-Higashi syndrome; c) granulomatous disorders; d) cyclic and other neutropenias; e) eosinophilia; and f) infectious mononucleosis; and normal volunteers.

Extensive studies are in progress relating to the pathogenesis of fever, granulocyte function, immune and other responses of humans and experimental animals to infections, antigens, etc.

Objectives:

1. To assess varied biologic responses of normal and a variety of patients to etiocholanolone.

2. To assess the responses of humans to single and multiple doses of endotoxin (endotoxin tolerance).

3. To intensively study groups of patients with such poorly defined illnesses as familial Mediterranean fever, recurrent fever, Chediak-Higashi syndrome, midline granuloma, Wegener's granulomatosis, cyclic and other neutropenias in an attempt to better delineate the clinical syndrome.

4. To study the mechanisms responsible for the release of endogenous pyrogen.

5. To characterize and purify leukocytic pyrogen.

6. To assess the effect of immunosuppressive agents on human diseases.

7. To explore the pathogenesis of the increased susceptibility to infection in a variety of diseases.

8. To study what humoral factors, if any, are responsible for the granulocytopenias seen in certain diseases of man.

9. To determine the marrow granulocyte reserves in neutropenic states and to perfect better tests for this parameter.

10. To study the effect of fever on drug metabolism.

11. To determine the effect of endotoxin and etiocholanolone on coagulation and iron metabolism in man.

12. To define the features of the beige mouse which are similar to the Chediak-Higashi syndrome of man.

13. To determine the susceptibility of beige mice to infection.

14. To assess the protective effects of endotoxin in mice challenged with various mycotic organisms and, if possible, to characterize the humoral and cellular factor(s) responsible for protection.

15. To investigate the reaction between endotoxin and lysates from the horseshoe crab.

16. To characterize the humoral factors responsible for endotoxin tolerance.

17. To investigate the growth of C. albicans in human sera.

18. Development of a new radioassay of granulocyte chemotaxis employing $^{51}Cr$-labeled granulocytes.

19. Study the contribution of the classical (C142) and alternate complement pathways to generation of chemotactic factors in health and disease.

20. Study divalent cation requirements and calcium fluxes of human granulocytes exposed to chemotactic factor.

21. Study generation of chemotactic factors from human plasma by activation of the clotting (kinin-generating) system.

22. Study the effects of dialyzable transfer factor on leukocyte chemotaxis.

23. Study in vitro leukocyte migration (chemotaxis) of normal and Chediak-Higashi mouse leukocytes.

24. Evaluate the effects of anticoagulants on in vitro studies of leukocyte migration.

Methods Employed:

Patients and volunteers receive varying doses of endotoxin and etiocholanolone. Rectal temperatures are taken and a variety of responses are determined: antibody levels, immunoglobulins, plasma cortisol, growth hormone, serum iron, clotting factors, etc.

The mobilization of peripheral granulocytes in response to etiocholanolone, endotoxin, or cortisol is studied in patients before, during and after chemotherapy.

Basic studies of immune mechanisms in dysproteinemias, Chediak-Higashi syndrome, etc., include quantitative immunoglobulin determinations using agar diffusion methods, immunoelectrophoresis, paper electrophoresis, complement levels, levels of antibody by the bentonite flocculation technique, and ability of individuals to develop delayed hypersensitivity through challenge with dinitrochlorobenzene on the skin.

Mice are given endotoxin and subsequently challenged at varying time intervals with such infectious agents as Candida albicans, Plasmodium berghei, etc. Donor mice are exsanguinated; the whole sera or fractions are passively transferred to normal mice, which are then challenged with the infectious agent. Iron is given to mice to study its protective role against infections.

An automatic temperature recorder is used to obtain rectal temperatures at regular intervals before and after challenge with antigen, complexes or pyrogen. Hematological responses are evaluated by analysis of ear vein blood samples obtained at various times preceeding and following challenge. Circulating pyrogen is assayed by passively transferring serum from challenged, immunized animals into normal recipients. Buffy coat leukocytes are separated from whole, heparinized rabbit blood following centrifugation and washed in phosphate buffered saline. Counts are made on aliquots of the washed leukocytes, and various concentrations of the agents under study are added to the cell suspensions. After incubation for a specific time interval, leukocytic pyrogen is measured by injection of supernatants into normal and endotoxin-tolerant recipient rabbits, and febrile responses are obtained.

Human peripheral leukocytes are incubated overnight with killed S. albus organisms. The resulting supernatant contains leukocytic pyrogen. This material is concentrated by means of alcohol precipitation, dialysis, column chromatography and fractionation, isoelectric focusing and electrophoresis.

Peripheral blood leukocytes were separated from heparinized whole blood by dextran sedimentation or Hypaque-Ficoll separation. Chemotaxis was evaluated using a radioassay developed in this laboratory employing $^{51}Cr$-labeled leukocytes and a double micropore filter Boyden chamber. Chemotactic factors were generated by incubating serum with either endotoxins or antigen-antibody complexes (complement-related factors) or with kaolin (kinin-related factors) and then purified by sephadex chromatography.

Patient Material:

Patients are admitted to the Clinical Center, as are normal volunteers, for these studies. In addition, patients from other Institutes participate in these studies.

Major Findings:

Patients with recurrent fever of unknown etiology have been found to represent a diverse array of clinical disorders.

Iron can reverse some of the protective effects of endotoxin when given to mice infected with C. albicans or Pneumococci.

Endotoxin causes a significant increase in factor 8 in human beings.

Prednisone by mouth or intravenous cortisol in addition to etiocholanolone and endotoxin are potent stimuli for the release of granulocytes from the marrow granulocyte reserves.

Cyclophosphamide has produced striking remissions and clinical improvement in patients with Wegener's granulomatosis, nephrosis and a variety of hypersensitivity diseases.

Radiation therapy has proven to be remarkably effective therapy for midline granuloma.

The growth of C. albicans in human sera is inversely proportional to the pH and directly related to the iron saturation in human sera.

The limulus lysate assay has been found to not be specific for endotoxin alone. Positive tests are produced by polynucleotides, thrombin, etc.

Fever reduces the rate of clearance of antipyrine from the blood. This model drug is metabolized solely by the microsomes of the liver.

Fever significantly lowers serum iron and the magnitude of the decrease is proportional to the elevation and the duration of the fever.

Chediak-Higashi mice are more susceptible to infection with C. albicans, S. aureus and D. pneumoniae than are control mice.

A simple, objective and rapid radioassay of human granulocyte chemotaxis employing $^{51}Cr$-labeled leukocytes has been perfected.

Kinetic analysis of chemotactic factor formation by complement activation of human serum demonstrated that the classical (C142) pathway proceeds rapidly (within ten minutes), whereas alternate complement pathway activation is characterized by a 20 to 30-minute latency. Such analysis is critical for detecting clinically important abnormalities, since after 60 minutes of generation time normal chemotactic activity may be present despite complete absence or inhibition of one complement pathway.

The major chemotactic factor generated by activation of complement in human serum is predominately, if not entirely, the cleavage product of C5, C5a.

Both calcium and magnesium are required and synergistically support optimal chemotaxis of human granulocytes.

Exposure of granulocytes to three different chemotactic factors results in calcium efflux out of a granulocyte and out of the cytoplasm and into a granule containing fraction. A lowering of cytoplasm calcium may be critical for initiating leukocyte locomotion.

Activation of Hageman factor in plasma with kaolin results in generation of factors chemotactic for human granulocytes. Plasma from a patient lacking prekallikrein was defective in such chemotactic factor formation; addition or prekallikrein corrected the defect.

Dialyzable transfer factor was demonstrated to be a potent chemotactic stimulus for human granulocytes and a weak chemotactic stimulus for mononuclear cells. Transfer factor from a patient with Sezary's syndrome, who was anergic, had no chemotactic activity. The chemotactic and transfer factor activities were inseparable by partial purification (sephadex chromatography).

The anticoagulants EDTA, oxalate and citrate prevented formation of complement-related chemotactic factors in standard anticoagulant concentration but had no effect on granulocyte chemotaxis. Heparin, in standard anticoagulant concentrations, has little or no effect of chemotactic factor formation but a reversible inhibitory effect on granulocyte locomotion.

Significance to Biomedical Research and the Program of the Institute:

The large number and wide variety of studies listed above provide considerable new knowledge dealing with host response mechanisms to a wide variety of clinical and experimental diseases and the pathogenesis of fever.

Proposed Course:

The above outlined studies are to be continued and some areas are to be expanded.

Honors and Awards:

Dr. Wolff served as a member of many committees of the NIH and the Infectious Diseases Society of America. He is presently the Chairman of the Medical Board of the NIH (for the third time). He served as visiting professor or lecturer at many institutions including the Philadelphia-Presbyterian Hospital, Philadelphia, Pennsylvania; Vanderbilt University School of Medicine, Department of Medicine, Nashville, Tennessee; Saint Barnabas Medical Center, Department of Medicine, Livingston, New Jersey; University of Pennsylvania Hospital, Department of Medicine, Philadelphia, Pennsylvania; and the University of Virginia, Department of Internal Medicine, Charlottesville, Virginia.

Publications:

1. Blume, R. S. and Wolff, S. M.: The Chediak-Higashi syndrome: studies in four patients and a review of the literature. Medicine 51: 247-280, 1972.

2. Herzberg, J. H. and Wolff, S. M.: Chronic factitious fever in puberty and adolescence: a diagnostic challenge to the family physician. Psychiatry in Med. 3: 205-212, 1972.

3. Song, C. S., Gelb, N. A., and Wolff, S. M.: The influence of pyrogen-induced fever on salicylamide metabolism in man. J. Clin. Invest. 51: 2959-2966, 1972.

4. Wolff, S. M., Kimball, H. R., and Clark, R. A.: The Chediak-Higashi syndrome. In Bergsma, D. (Ed.): Birth Defects: Original Article Series. The Fourth Conference on the Clinical Delineation of Birth Defects. Part XIV: Blood. Baltimore, The Williams and Wilkins Co., Vol. 8, 1972, pp. 69-73.

5. Blaschke, T. F., Elin, R. J., Berk, P. D., Song, C. S., and Wolff, S. M.: Effect of induced fever on sulfobromophthalein kinetics in man. Ann. Intern. Med. 78: 221-226, 1973.

6. Gallin, J. I., Clark, R. A., and Kimball, H. R.: Granulocyte chemotaxis: an improved in vitro assay employing $^{51}$Cr-labeled granulocytes. J. Immunol. 110: 233-240, 1973.

7. Simon, H. B. and Wolff, S. M.: Granulomatous hepatitis and prolonged fever of unknown origin: a study of 13 patients. Medicine 52: 1-21, 1973.

8. Elin, R. J.: Erythrocyte survival in magnesium-deficient rats. Proc. Soc. Exp. Biol. Med. In press.

9. Elin, R. J. and Wolff, S. M.: Effect of endotoxin administration on the growth of Candida albicans in sera from various species. Canad. J. Microbiol. In press.

10. Elin, R. J. and Wolff, S. M.: Endotoxin-induced non-specific resistance to infection--a possible mechanism. Proceedings of Non-Specific Factors Influencing Host Resistance. In press.

11. Elin, R. J. and Wolff, S. M.: Bacterial endotoxins. In CRC Handbook on Microbiology. Chemical Rubber Company. In press.

12. Elin, R. J. and Wolff, S. M.: Effect of pH and iron concentration on growth of Candida albicans in human serum. J. Infect. Dis. In press.

13. Gallin, J. I.: Radioassay of granulocyte chemotaxis. In Sorkin, E. (Ed.): Chemotaxis, its Biology and Biochemistry. Basel, Karger. In press.

14. Hochstein, H. D., Elin, R. J., Cooper, J. F., Seligmann, E. B., Jr., and Wolff, S. M.: Further developments of limulus amebocyte lysate test. Parenteral Drug Association Bulletin In press.

15. Kass, E. H. and Wolff, S. M. (Co-Editors): Proceedings of the International Endotoxin Conference, 1972. Supplement, J. Infect. Dis. In press.

16. Kimball, H. R., Wolff, S. M., Talal, N., Plotz, P. H., and Decker, J. L.: Marrow granulocyte reserves in the rheumatic diseases. Arthritis Rheum. In press.

17. Patten, E., Gallin, J. I., Clark, R. A., and Kimball, H. R.: Effects of cell concentration and various anticoagulants on neutrophil migration. Blood 41: 711-719, 1973.

18. Wolff, S. M.: Biological effects of bacterial endotoxins in man. In Proceedings of the International Endotoxin Conference, 1972. Supplement, J. Infect. Dis. In press.

19. Wolff, S. M.: Familial Mediterranean fever (familial paroxysmal polyserositis). In Harrison, T. R. (Ed.): Principles of Internal Medicine. Seventh Edition, New York, McGraw-Hill Company. In press.

20. Wolff, S. M.: Midline granuloma. In Harrison, T. R. (Ed.): Principles of Internal Medicine. Seventh Edition, New York, McGraw-Hill Company. In press.

21. Wolff, S. M.: Pathogenesis of fever. In Sanford, J. P. (Ed.): The Science and Practice of Clinical Medicine In press.

22. Wolff, S. M., Kimball, H. R., and Marshall, J. R.: The effects of hydrocortisone and estrogen on experimental etiocholanolone fever. J. Infect. Dis. In press.

23. Wolff, S. M. and Simon, H. B.: Granulomatous hepatitis and prolonged fever of unknown origin. Trans. Am. Clin. Climatological Assoc. In press.

24. Dale, D. C., Alling, D. W., and Wolff, S. M.: Application of time series analysis to serial blood neutrophil counts in normal individuals and patients receiving cyclophosphamide. Br. J. Hematol. 24: 57-64, 1973.

Serial No. NIAID-14(c)
1. LCI
2. Medical Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Medical Virology - Investigations of Host Response In Murine Coxsackie $B_3$ Myocarditis (Revised Title)

Previous Serial Number: SAME

Principal Investigator: Dr. Julius A. Kasel, until 11/1/72, then Dr. Michael S. Toren

Other Investigators: Dr. Michael S. Toren (until 10/31/72)
Mr. Holly A. Smith

Cooperating Units: NONE

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 2-6/12 | - | - | 2-6/12 |
| Professional: | 1-3/12 | - | - | 1-3/12 |
| Other: | 1-3/12 | - | - | 1-3/12 |

Project Description:

Objectives:

1. To define and evaluate immunologic factors which contribute to host recovery in coxsackie myocarditis.

2. To evaluate the effects of cortisone on coxsackie myocarditis.

Methods Employed:

Mice are inoculated with coxsackie $B_3$ virus to induce myocarditis. Animals are sacrificed at various periods of time after infection. Laboratory studies include histological examination of heart sections, neutralizing antibody titers, and myocardial virus titers. Anti-mouse thymocyte serum, cytoxan and cortisone are administered to evaluate their respective effects on the host response to infection.

Major Findings:

1. Anti-thymocyte serum does not aggravate the lesions of coxsackie

myocarditis. In fact, it ameliorates them.

2. Cytoxan aggravates the lesions of myocarditis, and suppresses antibody formation.

3. If cortisone is given at the same time as coxsackie, the lesions are aggravated. If cortisone is given 3 days after infection, the lesions are ameliorated, antibody titers are normal, and myocardial virus titers are normal.

Significance to Biomedical Research and the Program of the Institute:

1. The effects of anti-thymocyte serum and cytoxan on host response suggests that it is antibody production which is responsible for normal recovery from infection; and that perhaps the absence of antibody response is what leads to more chronic cardiomyopathies.

2. Cortisone administration to humans with coxsackie myocarditis is not contraindicated as previous animal studies have suggested. Previous investigators gave the drug simultaneously with the virus, whereas we have given the drug several days later, a situation more closely approximating the usual clinical circumstances.

Proposed Course:

The project will be terminated.

Honors and Awards: NONE

Publications:

1. Dolin, R., Blacklow, N. R., DuPont, H., Buscho, R. F., Wyatt, R. G., Kasel, J. A., Hornick, R., and Chanock, R. M.: Biological properties of Norwalk agent of acute infectious non-bacterial gastroenteritis. Proc. Soc. Exp. Biol. Med. 140: 578-583, 1972.

2. Murphy, B. R., Kasel, J. A., and Chanock, R. M.: Association of serum anti-neuraminidase antibody with resistance to influenza in man. New Eng. J. Med. 286: 1329-1332, 1972.

3. Rossen, R. D., Sell, K. W., Kasel, J. A., Shulman, N. R., Jora, J. J., Judd, K. P., Trentin, J. J., and Bulter, W. T.: Preparation of antilymphocyte globulin for human use: Attempts to find a combination of antigen cell dose and adjuvant which reliably provides immunosuppressive horse anti-lymphocyte globulin. Transplantation 14: 462-467, 1972.

4. Knight, V. and Kasel, J. A.: Adenovirus. In Knight, V. (Ed.): Viral and Mycoplasmal Infections of the Respiratory Tract.

Philadelphia, Pennsylvania, Lea and Febiger, 1973, pp. 65-86.

5. Knight, V. and Kasel, J. A.: Influenza virus. In Knight, V. (Ed.): Viral and Mycoplasmal Infections of the Respiratory Tract. Philadelphia, Pennsylvania, Lea and Febiger, 1973, pp. 87-123.

6. Couch, R. B., Kasel, J. A., Pereira, H. G., Haase, A. T., and Knight, V.: Induction of immunity in man by crystalline adenovirus type 5 capsid antigens. Proc. Soc. Exp. Biol. Med. In press.

7. Chalhub, E. G., Nusinoff, S. R., Kasel, J. A., Chanock, R. M., and Murphy, B. R.: Temperature sensitive mutants of influenza virus: III. Further characterization of the TS-1(E) influenza A recombinant (H3N2) virus in man. J. Infect. Dis. In press.

Serial No. NIAID-18(c)
1. LCI
2. Clinical Mycology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunology and Chemotherapy of Systemic Mycoses (Revised title)

Previous Serial Number: SAME

Principal Investigator: Dr. John E. Bennett

Other Investigators: Dr. Richard Diamond
Dr. Edward Block
Dr. Stephen Schimpff
Dr. Michael Frank
Dr. Joseph May
Dr. Michael Kane
Dr. John Gallin

Cooperating Units: Miss Anne Jennings, CP, NIH (Project No.63 )
Dr. Lee Henderson, University of Pennsylvania, Philadelphia, Pennsylvania
Dr. R. Gordon Douglas, Rochester University, Rochester, New York
Dr. William J. Klein, Jr., University of Alabama, Birmingham, Alabama

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 5 - 1/12 | | 1 | 6 - 1/12 |
| Professional: | 3 - 4/12 | | 1 | 4 - 4/12 |
| Other: | 1 - 9/12 | | -- | 1 - 9/12 |

Project Description:

Objectives:

1) Study lymphocyte-mediated responses to cryptococcal antigens in patients with cryptococcosis, in normal volunteers and in sensitized guinea pigs.

2) Study antibody and non-antibody mediated mechanisms for opsonization of cryptococci.

3) Evaluate chemotherapeutic effect of combined 5-fluorocytosine and amphotericin B in patients with cryptococcosis and in experimental murine cryptococcosis.

4) Determine serum protein binding and effect of hemodialysis on both amphotericin B and 5-fluorocytosine.

5) Elucidate the mechanism by which Cryptococcus neoformans achieves high level drug resistance during therapy with 5-fluorocytosine.

6) Develop a more sensitive and quantitative technique for the determination of anticryptococcal antibody and soluble circulating cryptococcal antigen by means of radioimmunoassay.

Methods Employed:

Peripheral blood cell separations. The following techniques were employed: human leukocytes-gravity sedimentation in plasma; human monocytes or neutrophils--Hypaque-Ficoll sedimentation; guinea pig leukocytes--sedimentation in 3% gelatin.

Lymphocyte transformation. Tritiated thymidine incorporation was tested by both tube and microtiter plate techniques using whole cryptococci, alkaline urea extracts of cryptococci, SK-SD, PPD-S and mumps antigen. Cell source was whole blood for guinea pigs and separated leukocytes for man.

Migration inhibition of peripheral leukocytes. Planimetry was used to determine the migration zone occuppied by leukocytes allowed to migrate 48 hours from a capillary tube into a chamber containing antigen. The migrating cell was not a neutrophil, as some have found, but appeared to be a mixture of lymphocytes and monocytes.

Opsonization of heat-killed cryptococci by serum, serum components or cerebrospinal fluid was measured by tumbling the microorganism with neutrophils for periods up to 2 hours. Extent of phagocytosis was determined microscopically. Several reagents were employed to discern the relative importance of different complement-mediated events to opsonization. The role of the alternate pathway was studied using sera in which the classical pathway was inoperative: inherited C2 deficiency of man, inherited C4 deficiency of the guinea pig, normal sera treated with EGTA, normal sera absorbed with cryptococci at 0°C, and absorbed sera reconstituted with properdin. Cobra venom factor was used to deplete late complement components (C3-9). Alternate pathway function was impaired using 50°C heating, withor without addition of C-2 after heating. Inactivation of both pathways was achieved by EDTA or 56°C heating. C3 on the surface of cryptococci was detected by fluorescent labelled goat antibody to C3.

Mechanism of resistance to 5-fluorocytosine was studied using incorporation of cytosine--$^{3}H$ and 5-fluorocytosine--$^{14}C$ into TCA soluble and TCA insoluble fractions of cryptococci.

Protein binding of drugs was measured by the ultrafiltration method of Bennett and Kirby.

Hemodialysis clearance of 5-fluorocytosine was measured in maintenance hemodialysis patients in Philadelphia following a single dose. Clearance of drug and endogenous serum creatinine were compared at different flow rates through the coil. Drug was measured by bioassay. Clearance of amphotericin B during hemodialysis was measured in patients undergoing therapy at Rochester, New York and Birmingham, Alabama.

Radiolabeling of purified cryptococcal polysaccharide was accomplished by coupling tyramine on the polysaccharide by cyanogen bromide. Tyramine was iodinated with $^{125}I$ using Chloramine -T.

Major Findings:

1) Both the lymphocyte transformation (LT) and migration inhibition of peripheral leukocyte (MIPL) techniques correlated with cutaneous delayed hypersensitivity reactions to cryptococcal antigens in guinea pigs and normal volunteers. Patients with cryptococcosis gave LT and MIPL values which were very reduced in comparison with skin test positive volunteers or sensitized guinea pigs.

2) An intact classical pathway of complement activation plus low levels of anticryptococcal antibody were necessary for serum to provide optimum kinetics for phagocytosis of cryptococci. However, opsonization of cryptococci was found to proceed primarily by the alternate pathway of complement activation. Purified cryptococcal polysaccharide in the absence of antibody could activate this pathway. Late complement components (C3-9) were essential for opsonization or generation of chemotactic factors.

3) Although serum from cryptococcosis patients opsonized cryptococci normally, cerebrospinal fluid (CSF) from these patients did not. Cryptococci recovered from CSF did not have detectable C3 on their capsular surface. These organisms, when incubated in the donor's serum, acquired C3 on their surface and showed marked changes in capsular morphology.

4) Guinea pigs depleted of late complement components by treatment with cobra venom factor died more rapidly after intravenous challenge with cryptococci than did controls ($p < 0.01$). The depleted animals also had larger numbers of cryptococci in blood and brain two days after challenge than did the control animals. This result supported the concept that terminal complement components are important in resistance to cryptococcosis.

5) Studies on human monocyte-derived macrophages, detailed in last year's report, were completed. Over two or more days, cryptococci within "activated" macrophages grew more rapidly than extracellular cryptococci. There were no detectable differences between macrophages from 12 normal subjects and 14 cryptococcosis patients.

6) Five-fluorocytosine was not bound to serum proteins whereas amphotericin B was more than 95% bound. In keeping with this observation, 5-fluorocytosine was readily removed by hemodialysis whereas essentially no amphotericin B was removed. Clearance of 5-fluorocytosine by the artificial kidney closely paralled creatinine clearance over a wide variety of flow rates.

7) Appearance of massive drug resistance during 5-fluorocytosine therapy was found due to mutational depression of UMP-pyrophosphorylase activity within the fungus in 5 of 6 such patients. In the sixth patient the mutation appeared to be in a cytosine-specific permease. A different mechanism of resistance was discovered in one in vitro induced mutant. The fact that this mutation reduced the growth rate and infectivity of the organism may explain why this mutation was not encountered clinically.

8) The combination of 5-fluorocytosine (5-FC) and amphotericin B was found to be at least additive in experimental murine cryptococcosis. Even low doses of amphotericin B significantly decreased the recovery of 5-FC resistant mutants from mouse brain.

9) A retrospective analysis of our experience with intraventricular amphotericin B therapy was made. The drug was administered to 21 patients with chronic meningitis. Implantation or usage of the siliconized rubber reservoir, by which means the drug was given, resulted in significant complications in 18 of the 21 patients. However, successful therapy was clearly attributable to intraventricular therapy in 3 of 15 cryptococcosis and 3 of 4 coccidioidomycosis patients.

10) Cryptococcal polysaccharide -$^{125}$I with high specific activity was prepared.

Significance to Biomedical Research and the Program of the Institute:

1) The two in vitro correlates of delayed hypersensitivity employed here indicated that lymphocytes from cryptococcosis patients have a defective response to cryptococcal antigens. This finding parallels results of others in another fungal infection, chronic mucocutaneous candidiasis. Just how this defect might influence resistance to infection is not clear with either infection. Although sensitized lymphocytes in the presence of cryptococcal antigen can "activate" human monocyte-derived macrophages, these macrophages are no better prepared to limit intracellular multiplication of cryptococci than are unactivated macrophages. Future attempts at restoring immune response to normal in cryptococcosis patients, for example with transfer factor, will depend on a clearer understanding of what the immune defects are and how they relate to recovery from infection.

2) The studies on complement components add to a growing body of evidence that serum, even devoid of specific antibody, can provide important factors for host resistance to infection. In cryptococcosis some of these factors, the result of the terminal sequence in complement activation, are

provided primarily through the recently described "alternate pathway." The studies raise the attractive hypothesis that some of the cardinal clinical features of cryptococcosis may be attributed to complement concentrations in the cerebrospinal fluid which are too low to generate opsonins or chemotactic factors. These features are predilection for the central nervous system, inability of the host to eradicate the organism once the brain is infected, and a poor cellular response in both infected brain and cerebrospinal fluid.

3) Amphotericin B or 5-fluorocytosine are given often enough to hemodialysis patients that it is clinically important to know the effect of dialysis on drug catabolism.

4) Our study of combined chemotherapy of cryptococcosis was prompted by the well-known toxicity of amphotericin B alone and our recently reported 60% failure rate with 5-fluorocytosine alone. Half the 5-fluorocytosine failures were due to drug resistance. Our mouse studies were sufficiently promising for us recently to initiate a controlled clinical trial, comparing the combination with amphotericin B alone.

5) Prior publications on reservoir insertion for intraventricular amphotericin B therapy have emphasized the efficacy and infrequent complications encountered. We hope to have provided a more sober, balanced appraisal of this mode of treatment.

6) Serologic tests for circulating soluble cryptococcal antigen in patient's body fluids are already known to have clinical value. Development of a radioimmunoassay for antigen may improve quantitation and sensitivity of this clinical tool.

Proposed Course:

1) A larger number of cryptococcosis patients will be studied at intervals during and after therapy to see how their clinical status correlates with the in vitro LT and MIPL response to cryptococcal antigens. Guinea pigs infected with Cryptococcus will be studied also. Patterns of defective response to in vitro testing will be examined in relation to the host's response to infection.

2) Preliminary studies have indicated that a soluble substance mediates the MIPL response to cryptococcal antigen. Hopefully, this observation will permit characterization of the mediator and dissection of defective MIPL response into two categories: defective elaboration of mediator and defective response of target cell to mediator. The possibility that this mediator is a chemotactic factor, acting to prevent migration, will be explored.

3) Future studies on the role of complement in cryptococcosis will concern a comparison of chemotactic factors generated in cerebrospinal fluid during cryptococcal versus purulent meningitis.

4) The clinical trial of combined 5-fluorocytosine and amphotericin B therapy for cryptococcal meningitis will continue. Other investigators are being contacted to see if they will engage in a collaborative study.

5) Radiolabeled cryptococcal polysaccharide will be purified by affinity chromatography to remove labeled but non-immunogenic components. Radioassay will be compared with available techniques for detection of anti-cryptococcal antibody and cryptococcal antigen.

6) Studies on the pharmacology and mechanism of resistance to 5-fluorocytosine will be discontinued.

Honors and Awards:

Dr. John E. Bennett has engaged in the following outside activities during the past year:

1) Invited to contribute five chapters on systemic mycoses for the next edition of "Cecil and Loeb's Textbook of Medicine."

2) Invited to serve again on the editorial board of "Control of Communicable Disease in Man", the 1975 edition.

3) Asked to continue to serve as a consultant to the Advisory Committee, Anti-infective Agents Division, Food and Drug Administration.

4) Continues to serve on the editorial board of the journal "Antimicrobial Agents and Chemotherapy".

5) Together with Dr. Fred Gill (also LCI) organized a monthly Inter-Institution Infectious Disease Rounds for the Washington metropolitan area.

6) Was an invited lecturer at the following medical schools: Cornell, Harvard, Johns Hopkins, Rochester, (N.Y.), University of Pennsylvania (Philadelphia), Mount Sinai (N.Y.) and others. Also an invited participant at the First International Symposium on Clinical Immunosuppression (New York City).

Dr. Richard Diamond:

1) Continued to serve as a member of the Advisory Committee, Anti-Infective Agents Division, Food and Drug Administration.

2) Recipient of the Anna Fuller Fund for study of cellular immunity at the Clinical Research Centre at Harrow, Middlesex, England.

3) Invited lecturer at Downstate Medical Center (SUNY), Michael Reese Hospital, and University of Chicago.

Publications:

1. Bennett, J. E.: Cryptococcosis. In Hoeprich, P. (Ed.): Infectious Diseases. New York, Harper & Row, Inc., 1972, pp. 945-952.

2. Block, E. R. and Bennett, J. E.: Pharmacological studies with 5-fluorocytosine. Antimicrob. Agents Chemother. 1: 476-482, 1972.

3. Young, R. C., Jennings, A., and Bennett, J. E.: Species identification of invasive aspergillosis in man. Am. J. Clin. Pathol. 58: 554-557, 1972.

4. Jennings, A. E. and Bennett, J. E.: The isolation of red yeast-like fungi in a diagnostic laboratory. J. Med. Micro. 5: 391-394, 1972.

5. Bennett, J. E., Bonner, H., Jennings, A. E., and Lopez, R. I.: Chronic meningitis caused by Cladosporium trichoides. Am. J. Clin. Pathol. 59: 398-407, 1973.

6. Block, E. R., Jennings, A. E., and Bennett, J. E.: Experimental therapy of cladosporiosis and sporotrichosis with 5-fluorocytosine. Antimicrob. Agents Chemother. 3: 95-98, 1973.

7. Diamond, R. D. and Bennett, J. E.: A subcutaneous reservoir for intrathecal therapy of fungal meningitis. New Eng. J. Med. 288: 186-188, 1973.

8. Block, E. R. and Bennett, J. E.: The combined effect of 5-fluorocytosine and amphotericin B in the therapy of murine cryptococcosis. Proc. Soc. Exp. Biol. Med. 142: 476-480, 1973.

9. Levine, A. S., Siegel, S. E., Schreiber, A. D., Hauser, J., Preisler, H., Goldstein, I. M., Seidler, F., Simon, R., Perry, S., Bennett, J. E., and Henderson, E. S.: Protected environments and prophylactic antibiotics: A prospective controlled study of their utility in the therapy of acute leukemia. New Eng. J. Med. 288: 477-483, 1973.

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15. Diamond, R. D. and Bennett, J. E.: Disseminated cryptococcosis in man: Decreased lymphocyte transformation in response to Cryptococcus neoformans. J. Infect. Dis. In press.

Serial No. NIAID-23 (c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Observations on Secretory Immunoglobulins (Primarily Canine, but Includes Rabbits and Guinea Pigs also) (Revised title)

Previous Serial Number: SAME

Principal Investigator: Dr. Herbert Y. Reynolds

Other Investigators: Dr. Douglas M. Levin

Cooperating Units: Dr. R. G. Mage, LI, NIH (Project No. NIAID 146)
Dr. D. L. Rosenstreich, LMI, NIDR, NIH

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 7/12 | | | 7/12 |
| Professional: | 4/12 | | | 4/12 |
| Other: | 3/12 | | | 3/12 |

Project Description:

Objectives:

1. To complete the molecular model for canine secretory IgA and identifying the assembly and interactions of secretory component (SC) and J-chain.

2. To elucidate the ultrastructure of secretory component and in vitro to study the susceptibility to proteolysis of dimeric serum and secretory IgA. Further, enzymes in gut epithelial cells (from in vitro cell cultures) responsible for synthesis of SC and for the assembly of SC and IgA are being sought.

3. To examine the gut associated lymphoid tissue (Peyer's patches) in dogs and guinea pigs for its response to bacterial antigens and for its capacity to process antigens and to produce immunoglobulins (antibody).

Methods Employed:

Gel filtration and ion exchange chromatography, affinity and immunoadsorbent chromatography, standard techniques of zone electrophoresis, immunodiffusion; sedimentation velocity and equilibrium ultracentrifugation;

equilibrium dialysis and fluorescence quenching for determination of binding energies; liquid scintillation spectroscopy; polyacrylamide disc gel electrophoresis. Intestinal lymphoid cell tissue cultures, autoradiography; immunofluorescence and stimulation of lymphocytes by mitogens including PHA, Con A, endotoxin et cetera, measuring $^{3}H$ thymidine incorporation or lymphokine production (MIF).

Major Findings:

Secretory component (SC), predominantly attached to colostral and intestinal IgA through convalent (disulfide) bonding, has been dissociated, and isolated and its molecular weight of approximately 50,000 daltons established. Further isolation of J-chain, using polyacrylamide separation techniques, from canine colostrum has been done. Further characterization of SC and J-chain with amino acid analysis and sequence awaits the purification of a larger amount of appropriate glycoprotein. Likewise, intestinal IgA (Reynolds and Johnson, J. Immunol.4: 888, 1970), is being prepared in batch quantities for use in proteolysis experiments and for use in antibody purification with Sepharose immunoadsorbents.

Peyer's patch lymphocytes have been isolated and successfully maintained in tissue culture from guinea pigs for periods exceeding 7 days. These experiments faced a formidable technical problem considering the bacterial contamination present on the gut surfaces. The lymphocytes have been identified as approximately 33-55% B-type and 40% T-type. Observations on gut associated lymphocytes are being extended to the tracheobronchial tree where similar lymphocyte aggregates are present.

Significance to Biomedical Research and the Program of the Institute:

These studies defining the molecular models of canine IgA's have made critical testing possible for the role of secretory piece in protecting secretory IgA from proteolysis and in a transport function. J-chain, likewise, is important in the assembly of IgA and probably IgM molecules. All these studies of canine IgA help to extend the homology of the mucosal secretory defense system in mammals and humans which is recognized as a major barrier for interaction with viral and bacterial micro-organisms by the host.

Studies on the function of gut lymphoid cells in birds and mammals, such as the Peyer's patch areas, are contradictory and may point to the tremendous species variations which will prevent easy generalities about function and significance. Therefore, each mammalian species will ultimately require study. The interaction of lymphoid cells which process antigens, such as gut bacterial antigens, and those which subsequently produce appropriate antibodies are likely to be vital in regulating the growth of normal intestinal flora and in the development of intestinal illnesses such as cholera and other diarrheal diseases. Now that these cells can be cultured in vitro and the various lymphocyte populations accurately identified, some of the confusion has been removed and hopefully more precise functions of these lymphoid aggregates can be found.

Proposed Course:

1. Further work on the structure of secretory piece and J-chain to show the interactions with IgA immunoglobulin and the elucidation of the chains' ultrastructure.

2. To elucidate the function of antigen processing cells in gut lymphoid tissue and to extend these observations to the bronchial associated lymphoid tissue.

Honors and Awards: NONE

Publications:

1. Reynolds, H., Carta-Sorcini, M., and Mage, R.: The immunoglobulins derived from a $V_H$-$C_H$ recombinant rabbit and its normal relatives. I. Measurements of heavy chain molecular weights and allotypes of colostral IgA. Immunochemistry. In press.

2. Levin, D. M., Rosenstreich, D. L. and Reynolds, H. Y.: Immunological responses in the gastrointestinal tract of the guinea pig. I. Characterization of Peyer's patch cells. J. Immunol. In press.

Serial No. NIAID-30(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Interaction of Antibody and Complement in the Production of Immune Damage

Previous Serial Number: SAME

Principal Investigator: Dr. Michael Frank

Other Investigators: Dr. Joseph E. May
Dr. Michael A. Kane
Dr. Robert Gerwin
Dr. John I. Gallin
Dr. Richard Diamond
Dr. John E. Bennett
Miss Thelma Gaither

Cooperating Units: Dr. Robert Clark, Harborview Hospital, Department of Medicine, Seattle, Washington
Dr. Ira Green, LI, NIAID, NIH (Project No. NIAID-142)
Dr. Harry Kimball, Internal Medicine Associates, Yakima, Washington
Dr. John VanBoxel, LI, NIAID, NIH (Project No. NIAID-142)

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 2 - 8/12 | | 5/12 | 3 - 1/12 |
| Professional: | 2 | | 5/12 | 2 - 5/12 |
| Other | 8/12 | | -- | 8/12 |

Project Description:

Objectives:

1. To determine the role that each of the various immunoglobulin classes play in complement mediated cytotoxic reactions and the role of the several pathways of complement activation in cytotoxic reactions and generation of biologically active products from complement components.

2. To evaluate the role of complement in immunologic diseases.

Methods Employed:

Quantitative complement component assay, preparation of intermediates in the complement sequence, various complement components on the surface of cells and purification of complement components and antibodies to the various classes are routinely used in this laboratory. These methods employ the use of DEAE cellulose and C-M cellulose, Sephadex, electrophoretic techniques and ultra-centrifugation for the purification of immunoglobulins and complement component proteins. Cellular intermediates in the complement sequence (i.e. cells sensitized with antibody and reacted with various components of complement) are prepared by our own modification of standard methods and are used for titration of complement components. Efforts are made to determine the interrelationships and efficiency of various complement components reacting with other components from the same species and cross species lines. Purified preparations of various antibodies, antigens and endotoxins are used to study immunologic responsiveness of a new strain of guinea pigs recently established in our laboratory with a total deficiency of C4, one of the early reacting components in the complement sequence.

Major Findings:

The C4 deficient guinea pig strain, developed in cooperation with Drs. Ellman and Green, has been used to study the pathogenesis of immune damage and the function of the various pathways of complement activation in the generation of biologically active complement fragments. It was demonstrated that the classical complement pathway is capable of the rapid generation of chemotactic factors from normal guinea pig serum. Activation of the alternate pathway leads to a marked kinetic delay in generation of these factors. It was demonstrated that both pathways lead to the generation of a similar complement fragment with chemotactic activity, C5a. More recently, these findings have been extended to studies of complement in human serum; the results obtained with human serum are similar. Again the marked kinetic delay noted with alternate pathway activation was evident.

Studies of the cytotoxic potential of C4 deficient guinea pig serum which has a complete block in classical pathway function because of the complement component defect have been performed. It was possible to show the existence of a new mechanism of complement activation in this serum, whereby C1, a classical pathway component, is required for activation of the alternate complement pathway. Only through activation of C1 on a cell surface, can the alternate pathway of complement action be activated and thereby damage cells. A similar lytic mechanism was discovered to be operative in human serum as well as guinea pig serum. Studies performed *in vivo* also demonstrated that the well known effects of endotoxin administration on clotting were mediated via the classical complement pathway. In animals with absent classical pathway function endotoxin injection neither shortened clotting time nor induced a thrombocytopenia. *In vivo* studies also further defined the function of complement in detoxification of endotoxin and showed that both complement pathways contribute to this function.

In vitro studies showed that damage to normal cells and tissues proceeded much more efficiently via activation of the classical rather than alternate complement pathway.

More recently studies have been performed of the detailed interaction of the two major pathways of complement activation. Studies were performed using an in vitro system which leads to the opsonization of cryptococcal organisms by complement. This in vitro system is thought to be directly related to the in vivo events in patients exposed to cryptococci. It was shown that both pathways of complement activation are important in opsonization of cryptococci and that the classical pathway acts primarily in this case to modulate the activity of the alternate pathway.

Significance to Biomedical Research and the Program of the Institute.

These experiments are leading to a better definition of the role of the various complement pathways in the production of immune damage and protection from infections. They are placing many aspects of host defense against bacterial infection on a molecular basis and help explain many of the phenomena in autoimmune diseases. This information is essential to the rational development of chemotherapy for these illnesses.

Proposed Course:

The biological role of the alternate and classical complement pathways will be explored in greater detail. The interaction between complement, endotoxins, and clotting factors will also be evaluated.

Honors and Awards:

Portions of these data were selected for presentation at the International Endotoxin Conference, the World Health Organization Conference on Non-Specific Factors in Host Defense, the annual meeting of the Association of American Immunologists and at the Second International Conference on Immunodeficiency Diseases in Man.

Publications:

1. May, J. E., Green, I., and Frank, M. M.: The alternate complement pathway in cell damage. Antibody-mediated cytolysis of erythrocytes and nucleated cells. J. Immunol. 109: 595-601, 1972.

2. May, J. E., Kane, M. A., and Frank, M. M.: Immune adherence by the alternate complement pathway. Proc. Soc. Exp. Biol. Med. 141: 287-290, 1972.

3. May, J. E., Kane, M. A., and Frank, M. M.: Host defense against bacterial endotoxemia. Contribution of early and late components of complement to detoxification. J. Immunol. 109: 893-895, 1972.

4. Colten, H. R. and Frank, M. M.: Biosynthesis of the second (C2) and fourth (C4) components of complement in vitro by tissues isolated from guinea pigs with genetically determined C4 deficiency. Immunology 22: 991-999, 1972.

5. Green, I., Shevach, E. M., Stobo, J., Frank, M., and Heperman, R.: B-cell, T-cell and monocyte origin of the guinea pig L2C leukemia mouse lymphomas and human leukemias. In Jankovic, B. D. (Ed.): Micro-environmental Aspects of Immunity. New York, Plenum Publishing Co., 1972, pp. 491-498.

6. Root, R. K., Ellman, L., and Frank, M. M.: Bactericidal and opsonic properties of C4-deficient guinea pig serum. J. Immunol. 109: 477-486, 1972.

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8. Shevach, E., Herberman, R., Frank, M. M., and Green, I.: Receptors for complement and immunoglobulins on human leukemic cells and human lymphoblastoid cell lines. J. Clin. Invest. 51: 1933-1938, 1972.

9. Sandberg, A. L., Snyderman, R., Frank, M. M., and Osler, A. G.: Production of chemotactic activity by guinea pig immunoglobulins following activation of the C3 complement shunt pathway. J. Immunol. 108: 1227-1231, 1972.

10. Gaither, T. A. and Frank, M. M.: Studies of complement-mediated membrane damage: the influence of erythrocyte storage on susceptibility to cytolysis. J. Immunol. 110: 482-489, 1973.

11. Kane, M. A., May, J. E., and Frank, M. M.: Interactions of the classical and alternate complement pathway with endotoxin lipopolysaccharide. J. Clin. Invest. 52: 370-376, 1973.

12. May, J. E. and Frank, M. M.: A new complement-mediated cytolytic mechanism--The C1-bypass activation pathway. Proc. Natl. Acad. Sci., USA 70: 649-652, 1973.

13. Van Boxel, J. A., Paul, W. E., Frank, M. M., and Green, I.: Antibody-dependent lymphoid cell-mediated cytotoxicity: role of lymphocytes bearing a receptor for complement. J. Immunol. 110: 1027-1036, 1973.

14. Frank, M. M., Ellman, L., Green, I., and Cochrane, C. G.: Site of deposition of C3 in arthus reactions of C4 deficient guinea pigs. J. Immunol. 110: 1447-1451, 1973.

15. Clark, R. A., Frank, M. M., and Kimball, H. R.: Generation of chemotactic factors in guinea pig serum via activation of the classical and alternate complement pathways. Clin. Immunol. Immunopathol. In press.

16. Frank, M. M., May, J. E., and Kane, M. A.: Contributions of the classical and alternate complement pathways to the biological effects of endotoxin. J. Infect. Dis. In press.

17. Frank, M. M., May, J. E., and Kane, M. A.: Biological interactions of complement with endotoxin: Studies in C4-deficient guinea pigs. Proceedings of the conference on Non-specific Factors Influencing Host Resistance. In press.

18. Clark, R. A., Kimball, H. R., and Frank, M. M.: Serum Chemotactic factors in an animal model of a complement deficiency state. Clin. Immunol. Immunopathol. In press.

Serial No. NIAID-33(c)
1. LCI
2. Clinical Allergy and Hypersensitivity Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Pathogenesis of Delayed Hypersensitivity

Previous Serial Number: SAME

Principal Investigator: Dr. Charles H. Kirkpatrick

Other Investigators: Dr. Predrag Ugrinski
Dr. John Rhodes
Dr. Eric Ottesen
Dr. John Gallin
Dr. Douglas Levin
Mr. Terrill Smith

Cooperating Units: Dr. David Rifkind, University of Arizona College of Medicine, Tucson, Arizona
Dr. Nils Axelsen, University Copenhagen, Copenhagen, Denmark
Dr. J. C. Meek, Kansas University Medical Center, Kansas City, Kansas
Dr. Lauren Pachman, Children Memorial Hospital, Chicago, Illinois
Dr. Robert Clark, Harborview Hospital, Department of Medicine Seattle, Washington
Dr. Ira Green, LI, NIAID, NIH (No project no.)
Dr. Samuel Wells, Surgery Branch, NCI, NIH (No project no.)
Dr. James Burdick, Surgery Branch, NCI, NIH (No project no.)

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 2-9/12 | | | 2-9/12 |
| Professional: | 1-9/12 | | | 1-9/12 |
| Other: | 1 | | | 1 |

Project Description:

Objectives (Sub-project A):

1. Characterization of cellular immunity in patients with chronic mucocutaneous candidiasis.

Methods Employed:

Peripheral blood lymphocytes from healthy normal volunteers have been studied in short term culture with antigens from C. albicans and phytohemagglutinin (PHA). The kinetics of dose-response relationships have been determined by labeling cells with $^{3}H$-thymidine and counting incorporated radioactivity. Because dose-response curves require large numbers of blood lymphocytes, we have undertaken characterization of the effects of cell numbers on the kinetics of in vitro lymphocyte proliferation. Varying numbers of cells have been cultured in various volumes of culture media with antigen and mitogen doses expressed as both μgm per cell and μgm per ml of fluid.

Production of the lymphokine, macrophage migration inhibition factor (MIF), has been studied in cultured peripheral blood lymphocytes from patients with chronic mucocutaneous candidiasis. This assay involves exposure of lymphocytes to antigens in vitro, collection of culture fluids which are concentrated, and assayed for their capacity to inhibit migration of guinea pig macrophages.

The in vitro studies are correlated with in vivo cellular immunity as determined by skin testing with 7 antigens known to elicit delayed cutaneous responses.

Major Findings:

Twenty-one patients with chronic mucocutaneous candidiasis have been studied. Delayed skin responses have identified three immunological categories. Five patients had normal delayed reactions to C. albicans as well as other antigens. Eleven patients had negative skin tests to C. albicans, but reacted to other antigens. Five patients were negative to all antigens in the panel and anergy was confirmed by their failure to become sensitized by topical application of 1-chloro-2,4-dinitrobenzene. One of these patients has an immunological profile similar to the Nezelof syndrome.

The experiments with cultured lymphocytes have disclosed further heterogeneity of the patients' immune responses. No patient with negative skin reactions produced MIF in vitro. Thirteen of the 16 skin test negative subjects did not show antigen-induced lymphocyte proliferation in vitro. However, in 3 patients these responses were normal.

Studies with PHA in lymphocyte cultures have also demonstrated heterogeneity of responses. Anergy patients generally have impaired PHA responses, while those patients in whom only the response to Candida was impaired had normal responses.

Our investigations have shown that the "microculture" method for lymphocyte cultures gives responses that are proportional to but of lower magnitude than the traditional (macro-) lymphocyte culture technique. Because the micro-method employs only one-tenth the number of lymphocytes of the macro-method, the effects of cell numbers and medium volume were examined. It was found that $10^{5}$ cells per ml in two ml of culture medium gave dose-responses that were

similar to those seen with $10^6$ cells per ml in two ml, and that the responses were significantly greater than those seen with $2x10^5$ cells in 0.2 ml. Thus we have been able to adapt this modification of the microassay to routine use in pediatric as well as adult patients.

Significance to Biomedical Research and the Program of the Institute:

Data presented from this laboratory have shown that many patients with chronic mucocutaneous candidiasis have abnormalities of cellular immunity. Furthermore, the abnormalities are not the same from patient to patient. The observations presented above have been synthesized into a model for the pathogenesis of the disease which is described later.

Proposed Course:

These investigations will be continued in new patients referred for our study of chronic candidiasis in order to characterize their cell-mediated immunity.

Objectives (Sub-project B):

Development of programs for treatment of chronic mucocutaneous candidiasis which is based on clinical and immunological features.

Methods Employed:

In our series of patients, candidiasis either began between infancy and the third year of life, or between the ages of 8-13 years. Those patients with "late-onset" diseases were frequently under treatment with antibiotics for disorders such as acne vulgaris' or furunculosis at the time of development of candidiasis.

We have proposed that patients with early onset disease have a defect in cellular immunity that has a congenital basis. In these patients, therapy must be directed at both eradication of the organism and restoration of intact cellular immunity. Patients with "late-onset" candidiasis may have a defect in host-defenses, but it has not been defined. From the results of the therapeutic trials, we believe that the immunologic abnormalities observed in this group of patients are acquired.

Amphotericin B has been used as the antifungal agent. The dosage range has been small, 800-1100 mgm, and administered over 7-8 weeks. When cultures from the nail folds become negative, the nails are avulsed in the belief that this is one of the reservoirs for recurrent infections.

Immunologic reconstitution has been done with transfer factor, a dialyzable substance obtained by disrupting leukocytes from skin test positive subjects. The optimal protocol for transfer factor is unknown, however, our protocol has been monthly injections for 3 months, then every other month. Recipients are monitored from in vivo skin responses and in vitro lymphocyte responses.

Major Findings:

The investigations conducted thus far have revealed additional defects in host defenses in patients with chronic candidiasis and have illustrated the importance of immunologic "staging" prior to implementation of a therapeutic program.

Five patients with "late-onset" candidiasis have received amphotericin B and nail avulsion. Four patients have remained free of disease for 3 1/2, 2 1/2, 2 and 8/12 years respectively. The fifth patient is currently receiving amphotericin. The two patients who were candida skin test negative have converted to positive skin tests.

Five patients have been treated with transfer factor alone. Although skin test reactivity and MIF production were observed in all five patients, there were no clinical responses.

Subsequently, two patients have been treated with the combined program of amphotericin B and transfer factor. Complete clearing of the mucocutaneous lesions occurred and both patients were in remission for several months. In one patient candidiasis of the tongue recurred after he was given erythromycin for a cold. This cleared with antifungal drugs but recurs periodically. The second patient was well for 9 months, but developed mucocutaneous recurrences when she began using an oral contraceptive. The lesions cleared with cessation of the pill and a short course of antifungal therapy.

One patient with a severe defect in cellular immunity received a thymus transplant. The procedure was followed by restoration of T-lymphocyte functions such as responses to phytohemagglutinin and concanavalin, E-rosette formation and acquisition of delayed-type skin responses. The reconstitutive effect have persisted for eight months but may be fading at this time.

In collaboration with Dr. Robert Clark,a patient with candidiasis, and recurrent pyogenic infections, anergy to candida and a unique defect in leukocyte chemotaxis has been studied. Her serum was capable of generating normal chemotactic stimuli, but her polymorphonuclear leukocytes would not respond. Transfer factor in a dose large enough to convert her delayed skin test did not correct the chemotactic anomaly.

Significance to Biomedical Research and the Program of the Institute:

From this experience it seems reasonable to assume that consideration of both clinical and immunological factors is essential for selection of effective therapy in candidiasis. Patients with "late-onset" disease may or may not have defective cellular immunity. The defect in these patients is apparently acquired and analogus to "immune-deviation" in experimental animals. Clearing of the fungal infection with amphotericin also reduces the tolerogenic load of antigen. In two cases the patients have become responsive to the antigens of candida, a event that would be expected in view of their obvious exposure to the antigen.

Proposed Course:

We plan to extend these treatment schedules to additional patients and continue the follow-up studies on the subjects who have been treated. As new treatment programs are developed they will be applied to the appropriate patients. An unresolved question with high priority is the importance of candida-specific transfer factor in treatment of chronic candidiasis.

Objectives (Sub-project C):

1. Characterization of the composition, properties and mechanisms of action of transfer factor.

2. Therapeutic trials with transfer factor in patients with defined immune deficiency syndromes.

Methods Employed:

The in vivo and in vitro tests of immunologic functions in man have been described in sub-project A. In addition, two major new endeavors have been added: characterization of a chemotactic agent in transfer factor and establishment of an animal model for transfer factor in the rhesus monkey.

Major Findings:

Reconstitution of immune functions in candidiasis as indicated by conversion of negative skin tests to positive has occurred in 5 recipients of transfer factor. Failures occurred in patients with Nezelof's syndrome. There has been good correlation between skin test reactivity and MIF production by cultured lymphocytes. In contrast, restoration of lymphocyte transformation responses has occurred only once. The immunologic basis of this disparity in candidiasis patients has not been established. It is of interest that similar findings have recently been reported in patients with the Wiskott-Aldrich syndrome. One possibility is that the lymphocytes which respond to antigens with thymidine incorporation and those which respond with lymphokine production comprise separate populations. If so, transfer factor preferentially affects the functions of the lymphokine producing cells. Alternatively, the differences may be due to differences in cell receptors which are dose dependent.

In collaboration with Dr. John Gallin, a chemotactic substance in transfer factor has been characterized. It is strongly chemotactic for polymorphonuclear leukocytes and weakly attractive for monocytes. It has a molecular weight of less than 5000 daltons and is not derived from complement. In brief, it apparently differs from previously described chemotactic factors. Preliminary studies have shown that the chemotactic activity and skin test-converting activity elute together from gel filtration columns.

A model for transfer factor activity has been established in rhesus monkeys. The transfers with transfer factor are specific but skin test sites must be biopsied for interpretation. The monkey model is currently being employed to monitor activity in column fractions and other experiments directed toward purification of transfer factor. Unfortunately, because we only have space for 4 monkeys, the progress in this project is disappointly slow.

Previous studies with transfer factor have been criticized because naturally occurring antigens were employed. This raised the possibility that transfer factor did not confer new immunologic information onto the recipient, but had an enhancing or adjuvant-like activity the facilitated expression of previous sensitizations. Transfer factor from a donor with delayed allergy to a synthetic antigen has been studied in 3 monkeys and 2 patients. In no case was delayed allergy to the new antigen transferred.

The transfer factor project culminated in an International Workshop on the Biological Properties and Clinical Applications of Transfer Factor in February, 1973. A copy of the Report of this meeting is attached (Appendix A).

Significance to Biomedical Research and the Program of the Institute:

These observations may be interpreted within the framework of the model for delayed hypersensitivity presented from this laboratory. The model envisions a multicellular system for antigen recognition and lymphokine synthesis analogous to that demonstrated for certain antibody responses in rodents. In the model, both antigen reactive cells and lymphokine producing cells would be "thymus-dependent".

The model therefore predicted that athymic patients with the Nezelof syndrome would not respond to transfer factor and this prediction has been substantiated in 3 cases.

The responses to transfer factor in the candidiasis patients may indicate that their immunological defect lies at a site that prevents expression of cellular immunity be lymphokine production. The lesion is apparently corrected or circumvented by transfer factor. Obviously, future experiments will be addressed to more precise delineation of the defect and the mechanism of action of transfer factor.

Proposed Course:

The experiments on purification and characterization of transfer factor will be continued.

Objectives (Sub-project D):

Search for linkage between chronic mucocutaneous candidiasis and histocompatibility antigens.

Methods Employed:

Peripheral blood lymphocytes have been typed for histocompatibility antigens by the microcytotoxicity assay. This work was done through an agreement with Dr. Paul Terasaki, UCLA Medical School.

Major Findings:

The unusual distributions of HL-A antigens in patients with chronic mucocutaneous candidiasis are summarized in the table:

| Antigen | Frequency (%) | |
|---|---|---|
| | Candidiasis Patients | Control |
| HL-A 3 | 52 | 18 |
| HL-A 9 | 13 | 23 |
| W 28 | 9 | 28 |
| HL-A 7 | 43 | 14 |
| HL-A 12 | 43 | 11 |
| HL-A 13 | 4 | 17 |
| W 8 | 13 | 5 |

Significance to Biomedical Research and the Program of the Institute:

Studies in animals have shown that responsiveness to certain antigens is genetically controlled and linked to major histocompatibility antigens. Subsequently, human studies have shown unusual frequencies of HL-A antigens in patients with SLE, leukemia, Hodgkins disease and non-tropical sprue.

The finding of an unusual frequency of HL-A in patients with candidiasis may be evidence for linkage of immunologic unresponsiveness in these patients to a hstocompatibility antigen. These studies will be pursued to clarify this point.

Proposed Course:

Although these studies will be extended to include new candidiasis patients as well as other patients with immune defects, the data on the 25 patients are currently being analyzed to see if the HL-A antigens identify a sub-group of patients with candidiasis.

Objectives (Sub-project E):

1. Characterization of serum antibody specificities in chronic mucocutaneous candidiasis.

2. Screening of patients sera for free candida antigens.

Method Employed:

Over 50 antigenic specificities have been identified in rabbit antisera to disrupted C. albicans. In collaboration with Dr. Nils Axelsen, the scientist who has identified these antigens, we have screened sera of candidiasis patients for their antibody profiles. The technique uses crossed antigen-antibody electrophoresis and gives both qualitative and quantitative data.

By using patients serum in place of the candida fractions, the system has been adapted for detection of free antigens in serum.

Major Findings:

The serological profiles of six patients have been completed and six additional patients are under study. The qualitative and quantiative responses divided the patients into 2 groups. One group had antibodies against many antigenic fractions and the titers were generally very high. The second group had few responses and low titers. Of particular interest was the fact that the patients with numerous and generally high-titered responses were those with the most extensive and severe cutaneous disease. Free candida antigens have been identified on only one patient.

Significance to Biomedical Research and the Program of the Institute:

The mechanisms of the extensive cutaneous inflammation in candidiasis patients is unknown. The defective cellular immunity makes this route of inflammation unlikely. However, the marked serological responses in these patients may indicate a role for antigen-antibody-complement complexes in the pathogenesis of this form of candidiasis. This postulate is supported by the finding of free candida antigens in the serum from one patient. The alternative explanation for the serologic findings is that these responses are secondary to chronic intradermal immunization with candida.

Confirmation of these models will be difficult but immunofluorescent studies of skin biopsies are planned.

Proposed Course:

These studies will be extended to the new candidiasis patients as they become available. Tissue studies of antigen, antibody and C are planned, and the response patterns will be compared to the HL-A typing.

Objectives (Sub-project F):

1. Identification of an immunologic lesion in a malnourished patient.

Method Employed:

The in vivo and in vitro tests of cell mediated immunity were employed to evaluate the abnormality in cellular immunity in a patient with malabsorption and hypogammaglobulinemia.

Major Findings:

Although the patient was anergic to all natural antigens and could not be sensitized with chlorodinitrobenzene, a normal inflammatory responses was demonstrated by a PHA skin test. His lymphocytes responded to antigens in vitro with thymidine incorporation but not MIF production. After correction of the malnorished state, his skin test became positive and MIF production was present. These data indicate that the immunologic defect in our patient was due to failure of the effector limb of immunity.

Significance to Biomedical Research and the Program of the Institute:

Susceptibility to infectious diseases is well-known among malnorished peoples. Identification of the nature of an immune defect in a malnorished subject has therapeutic potential - perhaps transfer factor would be beneficial in certain malnorished patients.

Proposed Course:

This investigation will be continued when additional, suitable subjects are referred for study.

Honors and Awards:

1. Extra-laboratory activities of the Principal Investigator -

   a) Chairman, NIAID Clinical Research Committee

   b) Project Officer, NIAID Contract with Allergy Foundation of America to establish criteria for diagnosis of allergic diseases

   c) Project Officer, NIAID Contract for purification of antigen E, Ra3 and K from short ragweed pollen

   d) Member, Special Study Section, NIAID Allergy Clinical Centers, 1973

   e) Member, Special Study Section, NHLI, Specialized Center for Research (SCOR) for pulmonary disease, 1972-1973.

f) Member, Weather and Air Pollution Committee and Audiovisual Committee. American Academy of Allergy

g) Chairman, International Committee, American Academy of Allergy

h) Editorial Board, Journal of Cutaneous Pathology.

Publications:

1. Kirkpatrick, C. H., Rich, R. R., and Smith, T. K.: Effect of transfer factor on lymphocyte function in anergic patients. J. Clin. Invest. 51: 2948-2958, 1972.

2. Kirkpatrick, C. H., Rich, R. R., and Smith, T. K.: Immunologic and clinical effects of transfer factor in anergic subjects. In Jankovic, B. D. and Isakovic, K. (Eds): Microenvironmental Aspects of Immunity. New York, Plenum, 1972, pp. 343-350.

3. Rich, R. R., Kirkpatrick, C. H., and Smith, T. K.: Simultaneous suppression of responses to allogenic tissue in vitro and in vivo. Cell. Immunol. 5: 190-200, 1972.

4. Clark, R. A., Root, R. K., Kimball, H. R., and Kirkpatrick, C. H.: Defective neutrophil chemotaxis and cellular immunity in a child with recurrent infections. Ann. Intern. Med. 78: 515-520, 1973.

5. Kirkpatrick, C. H.: Biological effects of transfer factor on lymphocytes from patients with chronic mucocutaneous candidiasis. In Braun, W. (Ed.): Non-specific Factors Influencing Host Resistance. Basel, S. Karger, 1973, pp. 287-294.

6. Kirkpatrick, C. H.: Delayed (cellular) hypersensitivity. In Speer, F., Dockhorn, R. J. and Shira, J. E. (Eds.): Allergy and Immunology In Children. Springfield, Ill., C. C. Thomas. In press.

7. Axelsen, N. H. and Kirkpatrick, C. H.: Simultaneous characterization of free candida antigens and candida precipitins in a patient's serum by means of crossed immunoelectrophoresis with intermediate gel. J. Immunol. Methods. In press.

8. Kirkpatrick, C. H.: Steroid therapy of allergic diseases. Med. Clin. N. Am. In press.

9. Kirkpatrick, C. H.: Immunology of chronic mucocutaneous candidiasis. Derm. Dig. In press.

10. Kirkpatrick, C. H., Wells, S. A., Burdick, J. F., and Smith, T. K.: Effects of fetal thymus transplantation on defective cellular immunity. In Good, R. A. (Ed.): Birth Defects Original Article Series. New York, New York, National Foundation. In press.

11. Kirkpatrick, C. H.: Restoration of cell-mediated immune responses with transfer factor. In Good, R. A. (Eds.): Birth Defects Original Article Series. New York, New York, National Foundation. In press.

12. Kirkpatrick, C. H., Meek, J. C., and Rich, R. R.: Mechanism of allergy to components of commercial bovine thyrotropin. J. Allergy In press.

APPENDIX A

ORGANIZERS' REPORT

Workshop on Basic Properties and Clinical Applications of Transfer Factor

The Transfer Factor Workshop was held at the Arizona Medical Center, Tucson, Arizona, on February 16-17, 1973. It was attended by approximately 90 scientists, including 11 representatives from 4 foreign nations. The general goals of the Workshop were to allow scientists working in this area of renewed activity to become acquainted with one another and to exchange views on the current problems and recent developments in this field.

In the opening remarks a number of pressing questions about transfer factor were presented. These included:

a) The cellular source of transfer factor. In most laboratories transfer factor is made from heterogeneous mixtures of leukocytes. It is important to know if transfer factor is a product of "T"-lymphocytes, B-lymphocytes or phagocytic cells such as monocytes and macrophages.

b) The mechanism(s) of action of transfer factor. Does transfer factor provide antigen-specific receptor sites to potentially responsive macrophages or lymphocytes? Could it act as a "messenger" which facilitates signal transmission from one cell to another? If so, which cell types are involved? Does transfer factor have less specific modes of action which permit expression of latent lymphocyte responses like lymphokine production?

A model for transfer factor must consider both specific and non-specific responses. What is the mechanism of the late appearance of some delayed skin reactivities which were not possessed by the transfer factor donor? Does this indicate that transfer factor has non-specific effects on cellular immunity as well as specific effects?

It has been reported that conversion of delayed skin responses occurs near the site of injection of transfer factor before it occurs at remote sites. What cellular events transpire during the interval between local and systemic transfer? What relationship do these events have to diseases such as sarcoidosis in which transfer factor induces sensitization at local sites but not systemically? Why has it been difficult to transfer contact allergy with transfer factor?

A related issue concerns the effects of prior skin tests and natural exposures to antigens on responses to transfer factor. What is the basis for the reported sequential transfer of delayed allergy from one transfer factor recipient to another? Studies with artificial and unnatural antigens and the appropriate animal models should help answer these questions.

c) The composition of transfer factor. Reports from various laboratories have suggested that transfer factor may be RNA, polypeptide or a small ribonucleoprotein. What are the relationships between dialyzable and non-dialyzable transfer factor to the immunologically-active RNA that has been extracted from lymphoid cells. Furthermore, what are the relationships between transfer-factors released from disrupted cells and the antigen-specific transfer factors liberated by incubating leukocytes with antigens?

d) In vitro and in vivo assays. Satisfactory in vitro assays and animal models for transfer factor have been difficult to develop. Many of the key questions concerning the mechanism of action of transfer factor can only be answered in experimental animals. An in vitro assay would be especially valuable for monitoring fractions obtained during purification experiments.

e) Potential therapeutic applications. Transfer factor is now under study in treatment of a variety of infectious, neoplastic and immunologically-deficient diseases. In order to evaluate the effects of transfer factor therapy in these patients a number of questions must be answered. These concern standardization of preparations, ascertainment of optimal doses and schedules of administration and determination of the importance of specificity (reactivity against a specific antigen) of the transfer factor used in a given disease.

Many of the above problems were addressed by the Workshop participants and by the end of the meeting many questions had been delineated and approaches to their solutions had been discussed. The following items are regarded as "high points" of the meeting:

I. Properties and Activities of Transfer Factor in vivo - Most participants agreed that administration of transfer factor was accompanied by conversion of delayed skin responses, and that usually only the donor's responses appeared in the recipient. Furthermore, after conversion of skin responses, the recipient's lymphocytes respond to specific antigenic stimulation in vitro with production of lymphokines such as macrophage migration inhibition factor (MIF). In contrast, it is rather uncommon for lymphocytes from the same subjects to respond to in vitro antigenic stimulation with enhanced thymidine incorporation (lymphocyte transformation). These observations are compatible with a hypothesis that different subpopulations of lymphocytes respond to antigens in different ways, i.e., some cells respond with DNA synthesis and cell division while others produce mediators such as lymphokines. The "target" for the action of transfer factor, either primarily or secondarily, could be the lymphokine-producing lymphocyte. An alternative explanation for the disparity in cellular responses would be that they are related to the doses of antigen or transfer factor employed. In fact, one investigator reported that lymphocytes from rhesus monkeys that had been given transfer factor responded to antigens with enhanced thymidine incorporation when the cultures were held for 7-8 days. In most laboratories, the cultures are harvested on the fifth or sixth day, so small but significant responses could have been missed.

II. Properties and Activities of Transfer Factor in vitro - The presentations during this session were especially exciting. An in vitro assay for transfer factor activity has been sought for years, and one laboratory presented data which indicated that they had both developed an assay and had determined why previous attempts had been unsuccessful. Apparently, the problem was loss of activity (or addition of an inhibitor) when transfer factor was dialyzed against commercial distilled water. This problem was obviated by dialyzing the transfer factor against tissue culture medium. The assay depended on the ability of transfer factor to "sensitize" peripheral blood lymphocytes from skin test negative subjects, so that they responded to antigenic stimulation in vitro with enhanced incorporation of thymidine into DNA. The assay was antigen-specific, and in this system transfer factor did not alter responses to mitogens such as phytohemagglutinin or endotoxin.

Other laboratories reported studies of MIF production as an assay for transfer factor activity. Lysates of blood leukocytes from hypersensitive subjects have been added to suspensions of cells from non-immune persons. Upon addition of antigen, MIF activity appeared in the culture fluids. It was of interest that the transfer factor-like activity in these lysates was not dialyzable, and therefore may be related to the RNA extracts which have been reported to exert similar effects.

The discovery of a new chemotactic substance in transfer factor was reported. This substance is dialyzable, enters the bed of sephadex G-25 and elutes in the same fraction as the skin test-converting transfer factor. Although it is strongly chemotactic for polymorphonuclear leukocytes, the material is not derived from complement or endotoxin. This property may provide a useful assay for tracing transfer factor activity through early steps of purification and fractionation.

Chemical characterization of transfer factor is under study in several laboratories. The active component resists degradation with ribonucleases and deoxyribonuclease, but is susceptible to pronase.

Sephadex G-25 gel filtration has been employed in several laboratories, but subtle differences in methodology made comparison of results somewhat tentative. In one laboratory, the skin test-converting activity and the chemotactic activity appeared in the same fraction. Others reported that the local skin test converting activity was concentrated in a different peak, however a pool of all other peaks also had activity.

Transfer factor fractionated on Sephadex G-10 yielded 3 peaks, the first of which (fraction A) was sub-divided into 3 more peaks ($A_1$, $A_2$ and $A_3$ by filtration through G-15. Cesium chloride banding indicated that peak $A_1$ was a polypeptide and peak $A_2$ was probably an RNA-polypeptide conjugate. Peak $A_2$ possessed skin test-converting activity, but the mixture of $A_1$ and $A_2$ did not. The active material ($A_2$) had an estimated molecular weight of approximately 2000 daltons and was believed to contain 34% RNA and 66% polypeptide. It would therefore contain 2-3 nucleic acid bases and about 12 amino acids.

III. Animal Models - Transfer factor-like reactions have been studied in various animals including guinea pigs, rats, rabbits and monkeys. There was considerable discussion of experimental designs and methods because some laboratories had been unable to confirm the work of others. For example, three workers described transfer factor-like activity in the supernatant fluids from incubated lymph node or peritoneal exudate cells from guinea pigs that were allergic to chlorodinitrobenzene (CDNB). One laboratory had not been able to transfer reactivity to tuberculin with analogous preparations, but preparations from guinea pigs allergic to CDNB were also active in rabbits and monkeys. Another lab reported transfer of sensitivity to PPD, coccidioidin and CDNB with the same types of culture fluids from lymphoid cells, as well as plasma from guinea pigs treated with anti-lymphocyte serum. Both workers emphasized that the experiments are not always repeatable and mentioned several problems such as inhibitors that they had encountered.

The results with monkeys were somewhat more consistent. Human transfer factor has successfully sensitized rhesus monkeys to PPD, candida, streptococci, KLH and mumps antigens. Monkey transfer factor also has been used to sensitize monkeys to PPD and schistosomal antigens. A model for transfer factor in the rat was also described. Rats treated with transfer factor from rats that were immune to Eimeria nieschulzi showed marked reduction in oocyst production and this phenomenon was correlated with delayed hypersensitivity.

Observations presented during the open discussion indicated that many transfer factor preparations also contain inhibitory substances which confuse interpretation of results. It seemed clear that development of acceptable animal models will be a substantial advance in this area and will permit studies with artificial antigens which cannot be done in humans.

IV. Standard Preparations - This subject was discussed at length although no decisions were reached. Many of the questions from the introductory comments were addressed: the cell of origin of transfer factor, possible differences between leukocytes obtained by plasmaphoresis or from a cell separator, characterization of donors, and standardization of preparations, dosages and schedules.

V. Clinical Studies - Transfer factor has been used in over 100 patients with a variety of immunologic, neoplastic and poorly understood inflammatory diseases. The responses have been variable and the differences in methodology have hampered comparison of one study to another. In many studies administration of transfer factor has been accompanied by clinical improvement by patients in whom conventional chemotherapy was unsuccessful. In spite of these positive observations, there were no totally controlled, objective studies of the therapeutic efficacy of transfer factor. The need for a blinded clinical trial with transfer factor in selected disorders was recognized by all of the clinical investigators. Ideally, this would employ standardized preparations of transfer factor from donors with known skin and in vitro responses, and would be prepared by one laboratory. The material would be given according to a uniform protocol with predetermined criteria for clinical responses. A general description of the general goals of a clinical trial and the diseases which could be studied is appended to the Resume'.

Nonetheless, the clinical observations in certain recipients of transfer factor were impressive:

a) Transfer factor has been given to over 20 patients with chronic mucocutaneous candidiasis. In most patients with this disease, a course of amphotericin B produces clearing of the lesions, only to have the infection recur in 2-6 months. Several candidiasis patients who received amphotericin B and transfer factor have shown sustained remissions during periods of observation of up to 18 months. In most laboratories, transfer factor alone had no clinical effects, although 2 investigators presented evidence to the contrary. The differences in clinical responses may be related to differences in transfer factor dosage.

b) A few patients with coccidioidomycosis and 1 anergic patient with tuberculosis had received transfer factor. In each case there had been no response to aggressive antimicrobial therapy, and transfer factor was added in despiration. Three of the coccidioidomycosis patients and the patient with tuberculosis showed marked clinical improvement on the combined program.

c) About 50% of patients with the Wiskott-Aldrich syndrome were judged to have fewer and less severe infections after treatment with transfer factor. A few cases even had improvement of the eczema and thrombocytopenia. In general, reduced incidences of infection occurred in patients whose monocytes lacked the receptor in IgG.

d) A number of patients with either combined immunodeficiency syndrome or thymus hypoplasia had received transfer factor. In most cases there were neither skin test conversions nor clinical responses. Again however, there were a few exceptions. The ability of the patients' lymphocytes to respond in mixed leukocyte reactions may predict skin test conversion by transfer factor.

e) Locally-injected transfer factor and CDNB had induced remissions in patients with multiple squamous cell carcinomas, and a number of patients with a variety of neoplastic disorders had apparent clinical benefits from transfer factor.

VI. Scientific Memoranda - The possibility of a Scientific Memoranda (SM) in this area was considered. The only reservations expressed by the participants were about the source of support. After being assured that the cost of the SM would not come from research funds or from monies that could support another workshop in the future, there was unanimous support for the SM. However, in view of the recent information that the SM would be terminated after one year, the value of the project seems dubious.

Finally, all participants were surprised by the number of investigators studying or using transfer factor. When the meeting was being planned in November and December, 1972, it was estimated that there would be 25-30 participants and 1 1/2 days would be adequate. Both estimates were too low. Unfortunately, there was not time for all participants to relate their work. Those scientists have been asked to submit resumes of their presentations which will be appended to the transcript of the recording of the Workshop.

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David Rifkind (by CHK)

David Rifkind, M.D., Ph.D.
Professor and Chairman
Department of Microbiology
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Serial No. NIAID-36(c)
1. LCI
2. Biologic Structure Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Structural Correlates of Antigen and Mitogen Interactions With Immunocompetent Cells

Previous Serial Number: SAME

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigators: Mr. J. Thomas Blake

Cooperating Units: Dr. Joseph M. Davie, Dept. of Pathology, Washington University Medical School, St. Louis, Missouri
Dr. J. Johnson, Scripps Clinic and Research Foundation, La Jolla, California
Dr. William E. Paul, LI, NIAID, NIH (Project No. 140)
Dr. D. L. Rosenstreich, NIDR, NIH (No Project no.)
Dr. Jack Stobo, Dept. of Med., Johns Hopkins Hospital, Baltimore, Maryland

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 6/12 | - | - | 6/12 |
| Professional: | 2/12 | - | - | 2/12 |
| Other: | 4/12 | - | - | 4/12 |

Project Description:

Objectives:

1. To develop general and specific cytochemical techniques which would permit visualization of antigen at the light and electron microscope level. Electrophoretically pure horseradish peroxidase and related peroxidases have been chosen for study because they will potentially permit both light and electron microscope study.

2. To correlate at a morphologic level cell interaction and the immunologic responses of such a model system.

3. To determine the cellular fate of antigen or mitogenic proteins in in vitro and in vivo models of cellular and humoral immunity using visible markers.

Methods Employed:

Electrophoretically pure horseradish peroxidase and cytochrome C peroxidase have been chosen as antigens with potential as visible immunologic markers. The following techniques have been applied to permit characterization of the immunobiology of these proteins as antigens and record the factors determining their use as detectable antigens at a cytochemical level:

1. Deoxyribonucleic acid synthetic response of lymph node, spleen, thymus and peripheral blood cells.

2. Nature and characterization of the antibody synthetic response in vitro and in vivo. A technique for simultaneous immunodiffusion studies and cytochemistry has been developed.

3. Isolation of anti-HRPO binding material from guinea pig serum by selective immune absorbent column chromatography and subsequent purification over Sepharose G-200 into Ig subclasses.

4. Specific antisera were raised against the Ig and anti-HRPO antibody and were characterized by Farr precipitation assays and hemagglutination studies performed by established and published techniques.

5. Preparation of antigenically active but enzymatically inactive derivatives are prepared by acid-acetone extraction of the heme group at -40°C, the heme moiety being required for enzymatic activity of the peroxidatic protein.

6. Horseradish peroxidase (HRPO) can be coupled to other immunogenic proteins by using gluteraldehyde as a cross-linking reagent at pH 7.0 and the derivative purified by starch block electrophoresis.

7. Antigen binding studies: Teased lymph node or spleen cells are washed three times in 1% serum-suspension media (Eagle's) and at a concentration of 100-200 million per milliliter incubated with or without antigen at various concentrations and at 4°C for 30-60 minutes. These cells are then washed 3-4 times over 100% fetal calf serum by density gradient and resuspended in 0.5 ml pure fetal calf serum and smeared on glass slides. Cells are air dried and fixed in either gluteraldehyde, methanol (-40°C) or acid-acetone (-40°C-70°C). After washing away fixative some slides are incubated with horseradish peroxidase and fixed in gluteraldehyde. Slides are then incubated in 3,3-diaminobenzydine in tris-HCl buffer at pH 7.6 and reaction product visualized after mordinification and dehydration for light microscopy.

Alternatively aliquots of gluteraldehyde fixed cells were processed for electron microscopy.

Major Findings:

1. Immunoglobulin and thus presumably antigen specific receptors exist tightly bound to the surface membrane of B lymphocytes. T lymphocytes by contrast lack easily detectable surface immunoglobulin. On interaction with antigen or antibodies directed against these determinants, migration of these receptors towards the centrosome associated cell pole occurs followed by internalization. While binding to these receptors occurs at 0-4°C, migration on contact with a multivalent ligand requires expenditure of cell energy for "capping" or internalization. However a clumping or aggregation of these prescribed receptors occurs within the plane of the cell membrane even in the presence of metabolic inhibitors. We have visualized at the ultrastructural level this phenomenon using a rabbit anti-guinea pig immunoglobulin reagent coupled to horseradish peroxidase (RAGIG-HRPO). Studies of the fate of internalized receptor with time is being pursued. (Work in collaboration with Drs. J. Davie and W. Paul).

2. In collaboration with Drs. J. Stobo and W. Paul we have investigated the interaction and fate of concanavalin A, a plant lymphocyte phytomitogen, with the target cell. EM studies as well as biochemical studies show a complex activation event both in terms of kinetics and differential responsiveness to Con A and PHA indicating heterogeneity of T-lymphocyte. Using Con-A-Cytochrome-C peroxidase complexes Con A was localized to patches of the cell surface.

3. An appreciable number (3-14%) of lymphocyte-like cells have been identified in mouse lymphoid cell populations which lack both detectable and membrane associated IgG surface determinants. These $\theta^-, Ig^-$ are not $\theta^-$ T lymphocytes and have ultrastructural features of prominent nucleoli and absent endogenous peroxidase activity. While these cells may be B lymphocytes with diminishing amounts of surface IgG, nonetheless they have features which suggest that they represent a distinct lymphocyte subset (in collaboration with Drs. J. Stobo and W. E. Paul).

Significance to Biomedical Research and the Program of the Institute:

A knowledge of structural correlates of the cellular events in immunologic interactions is necessary to fully comprehend such basic phenomena as transplantation rejection and immunoglobulin synthesis. Such an understanding in these areas would contribute greatly to the likelihood of therapeutic and pharmacologic manipulation of crucial host defense mechanisms. Macrophage migration appears clearly to be an *in vitro* correlate of cellular immunity and as such provides a model for studies of the ultrastructure and cellular correlation of delayed hypersensitivity.

Proposed Course:

1. Attempts to synthesize HRPO conjugates with mitogen (Concanavalin A) with Dr. W. E. Paul of the Laboratory of Immunology will be continued.

After initial characterization of the biochemical and immunologic properties of these compounds the localization of mitogen receptors and their fate in the in vitro proliferation response will be studied.

2. In collaboration with Dr. J. M. Davie, we will synthesize conjugates of rabbit anti-guinea pig immunoglobulin. Once synthesized the immunoglobulin membrane surface on guinea pig lymphocyte receptors will be mapped and characterized as to long term fate in vitro.

3. The fate of antigen binding cells using conjugates of HRPO and dinitrophenyl guinea pig albumin (DNP-GPA) or radioactive DNP-GPA will be characterized and their light and ultrastructure explored.

a. Determine the kinetics of the appearance of antigen binding cells (ABC) and antibody forming cells (AFC) and explore the localization of antigen at the level of the electron microscope.

b. Quantitation of receptors per cell and its relationship to the immunocompetence of the animal.

c. Attempt to differentiate antibody forming cell precursor from antibody binding cells and their origin (thymic or marrow).

Honors and Awards: NONE

Publications:

1. Stobo, J. D., Rosenthal, A. S., and Paul, W. E.: Functional heterogeneity of murine lymphoid cells. V. Lymphocytes lacking detectable surface or immunoglobulin determinants. J. Exp. Med. In press.

2. Rosenthal, A. S., Johnson, J. S., Rosenstreich, D. L., and Blake, J. T.: Evidence for a non-precipitating antibody in the guinea pig. Int. Arch. Allergy 44: 249-259, 1973.

3. Rosenthal, A. S., Davie, J. M., Rosenstreich, D. L., and Cehrs, K. U.: Antibody-mediated internalization of B lymphocyte to surface membrane immunoglobulin. Exp. Cell. Res. In press.

Serial No. NIAID-40(c)
1. LCI
2. Clinical Parasitology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Clinical Studies on Patients with Known or Suspected Parasitic Diseases

Previous Serial Number: SAME

Principal Investigators: Dr. Franklin A. Neva
Dr. Peter T. Franck
Dr. Theodore E. Nash

Other Investigator: Dr. Michael M. Frank

Cooperating Units: Dr. David W. Alling, OSD, NIAID, NIH (no project no.)
Dr. Richard Glew, LPD, NIAID, NIH (Project nos.133-L, 134).

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 1-8/12 | | | 1-8/12 |
| Professional: | 1-8/12 | | | 1-8/12 |
| Other: | -- | | | -- |

Project Description:

Objectives:

1) Study pathophysiology of parasitic diseases.

2) Develop improved criteria to assess severity or extent of disease, indications for treatment, and reversibility of disease process.

3) Contribute to training of clinical associates, guest workers, and medical community in general concerning clinical diagnosis and management of parasitic diseases.

Methods Employed:

Clinical contacts with various medical facilities continue to be made to establish our interest in clinical parasitology. Increasing numbers of patients, many of them NIH employees, are being seen (mainly in the OPD, but occasionally in the hospital) for evaluation of illness or symptoms after recent return from tropical environments.

The schistosomiasis patients are being studied immunologically for circulating antigen and antibody with gel diffusion and immunoelectrophoresis procedures with reagents prepared from infected animals. Standard blood smears and methods for determination of complement levels are being used for study of malaria patients.

Major Findings:

Most of the patients admitted to the LCI over the past year in connection with this project were either Peace Corps personnel or African students with schistosomiasis. Amebiasis and toxoplasmosis constitute the other major parasitic disease problems seen in the OPD or in-house. Although the followup experience with cases treated with Astiban is small, a surprisingly high failure rate (two of four cases) was found in S. hematobium infections. Dr. Nash's tests for circulating schistosome antigen or antibody to this antigen have disclosed antibody only in three of eight lightly infected patients with either S. mansoni or S. hematobium infections and neither antigen nor antibody in the sera of 27 heavily infected Brazilian cases. Dr. Nash's work has concentrated primarily upon characterization of the circulating antigen and is reported separately (Project no. 121-K of LPD, NIAID).

Only one new malaria patient was studied for serum complement alterations and for more detailed characterization of the time course of his parasite cycle. The malaria complement work has been extended to a monkey model and is being studied by Dr. Glew of LPD, NIAID and Dr. Atkinson of LCI, NIAID and will be reported separately (Project no. 133-L of LPD, NIAID). The results of malaria complement studies in the LCI patients and in prisoner volunteers are reported in LPD Project no. 134 and are being prepared for publication.

A number of Clinical Center patients were seen in consultation during the past year for diagnosis and/or management of known or suspected parasitic infections.

Significance to Biomedical Research and the Program of the Institute:

The research and service activities of this Section complement the various disciplines within the LCI and help provide a well-balanced training and research program in infectious diseases. The malaria studies did result in a separate collaborative project with Dr. Michael Frank's group on complement alterations in simian malaria. Further collaboration is being explored.

Proposed Course:

Dr. Peter Franck, who has been the main on-site representative of this Section in the Clinical Center, will be leaving the NIH at the end of fiscal year 1973. Dr. Theodore Nash will be available to assist Dr. Neva for clinical consultation and patient care in the parasitic disease area. The inability to plan for a senior investigator to replace Dr. Franck on a longer term than a one-year basis seriously inhibits development of this program.

Honors and Awards: NONE

Publications:

1. Franck, P. T.: Significance of geographic distribution of VEE virus variants. In Venezuelan Encephalitis. Proceedings of the Workshop Symposium on Venezuelan Encephalitis Virus. Washington, D. C., Pan American Health Organization, Scientific Publication No. 243, 1972, pp. 322-328.

Serial No. NIAID-44(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

**Project Title:** The Pathophysiology of Autoimmune Hemolytic Anemia

**Previous Serial Number:** SAME

**Principal Investigator:** Dr. Michael Frank

**Other Investigators:** Dr. Michael Kane
Dr. John Atkinson

**Cooperating Units:** Dr. Richard Root, University of Pennsylvania, Philadelphia, Pennsylvania
Dr. Alan Schreiber, Robert Breck Brigham Hospital, Boston, Massachusetts

**Man Years:**

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 1 - 10/12 | | | 1 - 10/12 |
| Professional: | 1 - 3/12 | | | 1 - 3/12 |
| Other: | 7/12 | | | 7/12 |

**Project Description:**

Objectives:

To define the pathophysiologic function of antibody and complement on the surface of red cells in patients with acute hemolytic anemia and to determine the effect of these substances on red cell survival. To determine how steroid therapy and splenectomy influences the survival of erythrocytes sensitized with antibody and complement. To this end a model of immune clearance of erythrocytes has been established in the guinea pig.

Methods Employed:

Rabbits have been immunized with guinea pig erythrocytes utilizing a number of different routes of injection, and IgM and IgG immunoglobulin fractions of high purity have been prepared from the rabbit antisera. Fractions containing antibody with high avidity for guinea pig erythrocytes have been used to sensitize guinea pig erythrocytes. The number of complement fixing sites per erythrocyte formed by these antibodies has been determined by use of the Cla fixation and transfer test. The antibodies have

been used to sensitize guinea pig erythrocytes labeled with chromium 51 and the half life of the cells studied after reinjection into guinea pigs.

In the most recently completed studies, guinea pigs were either treated with daily hydrocortisone acetate or subjected to splenectomy prior to clearance studies to determine the effect of these 2 therapeutic modalities.

Major Findings:

These studies have shown that IgM and IgG antibodies directed at cell surface antigens activate different mechanisms of clearance. In the case of IgM, antibody coated erythrocytes are sequestered by the liver, held in the liver for a period of time and then released back into the circulation as Coombs positive cells. IgG causes progressive splenic sequestration of the cells with no release back into the circulation once the cells are trapped.

Cortisone acetate acts to lower rates of sequestration of IgG and IgM sensitized cells. The reticuloendothelial system of treated animals behaves as if it had a lowered sensitivity to the complement coated cells. The kinetics of sequestration of the various cell types is unchanged in the cortisone treated animals as is the organ of localization. Also shown is that cortisone takes 4-6 days to produce a therapeutic effect. The effectiveness of the drug is dose related. Moreover, at low numbers of complement fixing sites/cell the drug is more effective than at high numbers.

Splenectomy on the other hand acts to reduce clearance of only the IgG coated cells. IgG coated cells are removed in the liver in splenectomized animals. The liver has one-sixth the activity of the spleen in removing cells with 17 IgG C1 fixing sites per cell. At increasing levels of sensitization the difference between splenectomized and normal animals becomes less and less marked and at 90 sites/cell the liver removes all the sensitized cells in both groups of animals. The kinetics of clearance of IgG coated cells by the liver are typical of those noted for IgG. Thus the class of antibody rather than the organ responsible for the sequestration determines the pattern of clearance obtained.

These studies are now being extended to examine clearance in animals with an activated reticuloendothelial system, such as might be noted in patients following a bacterial or viral infection. They are also being extended to human beings.

Finally, as a byproduct of these studies, it has been noted that cortisone therapy of animals has a marked effect on serum complement component levels. This effect takes two weeks to be maximally noted and is associated with a marked depression of some but not all components of complement. At low levels of cortisone, component titers (especially C1) rise. At higher levels C1 and C9 are unchanged but all other complement components undergo a profound fall in titer. For example at 20 mg/kg/day C4 and C3 titers halve in cortisone treated animals.

Significance to Biomedical Research and the Program of the Institute:

These studies define the role of antibody and complement in clearance of RBC's in patients with the disease, autoimmune hemolytic anemia. They represent the first molecular studies of the effect of immunoglobulin and complement on the survival of erythrocytes. They explain the finding, known for many years, that patients with hemolytic anemia may have Coombs positive cells in their circulation, which have a perfectly normal survival. Presumably, the cells have complement components placed on their surface by IgM. The studies point out the in vivo importance of earlier studies from this laboratory, demonstrating that IgM and IgG handle complement components differently and produce different kinds and amounts of damage to cell surfaces. The studies provide an explanation for the finding that some patients with hemolytic anemia respond to splenectomy and others do not and provide an explanation of the therapeutic response of patients to treatment with cortisone. Moreover they lay the ground work for an understanding of the effect of cortisone therapy on the severity of many illnesses in which tissue damage is thought to be a consequence of complement activation. In these cases the effect of cortisone therapy may be to profoundly depress the circulating level of many components of complement.

Proposed Course:

Studies of the effect of cortisone therapy and splenectomy on cell clearance are completed. Studies of reticuloendothelial activation and clearance are in progress as are studies of immune clearance in normal human beings.

Honors and Awards:

An abstract of this work was selected for presentation at the annual American Hematology Society Meeting, December 1972.

Publications:

1) Atkinson, J. P., Schreiber, A. D., and Frank, M. M.: Effects of corticosteroids and of splenectomy on the immune clearance and destruction of erythrocytes. J. Clin. Invest. In press.

2) Aptekar, R. G., Atkinson, J. P., Decker, J. L., Wolff, S. M., and Chu, E. W.: Bladder toxicity with chronic oral cyclophosphamide therapy in non-malignant disease. Arthritis Rheum. In press.

Serial No. NIAID-45(c)
1. LCI
2. Biologic Structure Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Ultrastructural Correlates of the Cellular Events in Host Defense Mechanisms in Man and Experimental Animals

Previous Serial Number: SAME

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigators: Dr. David C. Dale
Dr. Anthony S. Fauci
Dr. Michael M. Frank
Dr. Sheldon M. Wolff
Mr. J. Thomas Blake

Cooperating Units: Dr. Robert G. Horn, Dept. of Pathology, Vanderbilt University, Nashville, Tennessee
Dr. David L. Rosenstreich, NIDR, NIH (No Project no.)
Dr. Robert E. Scott, University of Minnesota School of Medicine, Dept. of Pathology, Minneapolis, Minnesota
Dr. Ethan M. Shevach, LI, NIAID, NIH (Project No. 142)
Dr. John van Boxel, LI, NIAID, NIH (Project No. 141)
Dr. Burton C. West, Dept. of Medicine, Vanderbilt University, Nashville, Tennessee

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 10/12 | - | - | 10/12 |
| Professional: | 5/12 | - | - | 5/12 |
| Other: | 5/12 | - | - | 5/12 |

Project Description:

Objectives:

1. Provide ultrastructural analysis of the cellular events in clinical disorders of the host differences in man and experimental animals.

2. Develop new morphologic techniques for investigating animal models of human disease.

3. Provide support for other investigators in their clinical and experimental approaches to human disease.

Methods Employed:

Methodologic approaches are as varied as the disorders to which they have been applied but in general involve fixation of tissue specimens or material provided by the collaborating units. These specimens are subjected to autoradiographic, cytochemical, ultrastructural and morphometric analysis by standard techniques. Where specific methodologies have been specifically developed, they will be briefly described under subject headings reviewed under Major Findings.

Major Findings:

1. A defect of cell maturation has been detected at the stage of 2nd granule formation in the developing marrow neutrophil. The appearance of such a lesion corresponds to the cyclic fluctuation of marrow differentiation in the grey collie. This defect, cytoplasmic autophage, glycogen aggregate deposits, accumulation of membrane vesicles suggest an autoregulatory defect of genetic origin in these dogs.

2. A biochemical analysis of gradient separated granules has been subjected to a corresponding electron microscopic analysis. Micrographs of granule fractions correlated morphologically with segregation of enzymatic activity into distinct classes. Interestingly, alkaline phosphatase activity in human WBC appears to be associated not with a granule but with a cell membrane fraction.

3. Immune complexes have been observed in 2 cases of Wegener's granulomatosis. Focal glomerular lesions are also present in the remaining cases examined ultrastructually. A correlation in the clinical severity of the disease process and the degree of functional renal involvement with the EM findings was observed.

Significance to Biomedical Research and the Program of the Institute:

1. Complete studies in progress.

2. Expand areas of promise commenserate with the available material.

3. Suggest from electron microscopic data new biologic approaches to problems already under study.

Proposed Course:

We will continue to provide collaborative facilities for LI and LCI/NIAID.

Honors and Awards: NONE

Publications:

1. McGee, Z. A., Ratner, H. B., Bryant, R. E., Rosenthal, A. S., and Koenig, M. G.: An antibody-complement system in human serum lethal to L-phase variants of bacteria. J. Infect. Dis. 125: 231-242, 1972.

Serial No. NIAID-46(c)
1. LCI
2. Biologic Structure Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunobiologic and Ultrastructural Characterization of the Mechanism of Antigen Recognition (Revised Title)

Previous Serial Number: SAME

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigators: Dr. James E. Balow
Dr. Jerrold J. Ellner
Dr. Dirk K. Greineder
Dr. Peter E. Lipsky
Mr. J. Thomas Blake

Cooperating Units: Dr. Ethan Shevach, LI, NIAID, NIH (Project No. 142)

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 3-2/12 | - | - | 3-2/12 |
| Professional: | 2-9/12 | - | - | 2-9/12 |
| Other: | 5/12 | - | - | 5/12 |

Project Description:

Objectives:

1. To develop and apply techniques for the isolation of immunocompetent thymus-derived lymphocytes.

2. To characterize in a quantitative and qualitative fashion the response of such cells to a variety of in vitro correlates of cellular immunity such as antigen-dependent lymphocyte proliferation and macrophage inhibitory factor production.

3. To characterize the ultrastructure and physiology of these cells as contrasted with other types of lymphocytes.

4. To study the mechanism of lymphocyte recognition and activation by antigen and mitogen using visible antigenic markers such as horseradish peroxidase (HRPO), HRPO-protein conjugates and radiolabelled antigens.

5. To study the regulation of cellular events and the role of cell cooperation in cellular immunity.

6. Characterize the role of macrophages in cell activation.

7. To study the mechanisms by which corticosteroids alter cellular immunity.

Methods Employed:

1. Determination of DNA synthesis of lymphocytic populations with and without addition of antigen by 4 hour pulse of $^{3}$H-TdR incorporation. Results assayed by either/or both autoradiography or liquid scintillation spectrometry.

2. Preparation and isolation of partially purified population of lymphocytes from oil induced peritoneal exudates or regional draining nodes subsequent (2-4 weeks) to immunization of inbred strain 2 and 13 guinea pigs with chemically defined antigens such as horse spleen ferritin, horseradish peroxidase or hapten protein conjugates.

3. Column purification: Peritoneal exudate cells (PEC) or lymph node cell suspensions were passed over glass bead columns and incubated at 37°C for 1 hour. Cells were then eluted with 10% guinea pig sera in minimal essential Eagle's media and the recovered cells studied.

4. Ultrastructural analysis of antigen localization by means of autoradiography (isotopically labelled antigen) or cytochemistry (peroxidase coupled or direct use).

5. Preparation of macrophage-rich oil-induced peritoneal exudate cells.

Major Findings:

1. Complex lymphoid cell mixtures placed over glass bead columns are depleted of antibody forming cells and their precursors as measured by loss of plaque forming cells (PFC'S); immunoglobulin and antigen specific receptor bearing cells as measured autoradiographically. By contrast those cells which participate in in vitro correlates of cellular immunity such as antigen-dependent lymphocyte proliferation and elaboration of macrophage inhibitory factor (MIF) are excluded from the column.

2. There appears to be within the lymphoreticular system a segregation both functionally and anatomically by immune competence. Those cells which mediate cellular immune function appear to be sequentered within inflammatory sites such as oil-induced peritoneal exudates. These sequestered cells possess enhanced immune responsiveness producing greater inhibition of macrophage migration by a factor of 10 than an equivalent number of lymphocytes derived from the region nodes. Moreover, antigen recognition as indicated

by new DNA synthesis in inflammatory exudate lymphocytes is 30-200 fold greater than an equivalent number of lymph node lymphocytes. The differences cannot be explained by presence or absence of macrophages, significant blastogenic factor release or desparities in the kinetics of cell responsiveness.

3. The peritoneal exudate lymphocyte have unique surface membrane changes on ultrastructural analysis.

4. The lymphocytes within the peritoneal exudate (PELS) appear to represent thymus-derived or T-lymphocytes. At least two types exist. One possesses a cell surface membrane modification, a uropod, whose formation is metabolically dependent. B lymphocytes which bear surface immunoglobulin do not appear to form uropods.

5. Activation of immunocompetent T lymphocytes is a complex phenomenon. While the exact mechanism is not established our data strongly indicate a multi-step event in which an initial interaction of antigen with an immunologically naive macrophage occurs followed by T-cell macrophage interaction and thence antigen recognition. T lymphocytes do not themselves appear capable of direct interaction with soluble antigen. Regression analysis of cell density and studies employing metabolic inhibitors indicate a necessary physical interaction between the immunospecific T-lymphocyte and the macrophage being immunorelevant antigen.

6. The mechanism by which corticosteroids block the action of a mediator of cell-mediated immunity macrophage inhibitory factor (MIF) has been studied. We have shown that this inhibition was not due to the inability of lymphocyte to recognize antigen or produce MIF but rather due to a block of the effect of MIF on its target cell the macrophage.

7. The activation of antigen dependent T lymphocyte proliferation has been shown to require an obligatory presentation of antigen on macrophages rather than direct antigen interaction with the immunospecific T lymphocyte. Moreover studies with soluble protein antigens has demonstrated that histocompatibility linked membrane determinant play a rate limiting role in macrophage lymphocyte cooperation.

8. Macrophages have been shown to possess surface membrane receptors from lymphocytes. Characterization of the kinetics, cellular specificity and ultrastructure of this phenomenon suggest that it may play an important role in immunologically relevant cell cooperation and in cellular differentiation and maturation.

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of an enriched population of cellular immune effector cells should provide a useful system for study of

the role of the cellular immune effector cells in immunologic surveillance and their contribution to the humoral limb of host defenses. Tumor immunity and transplantation immunity have been shown to rely to a great extent upon mechanisms of cellular immune function such as cytotoxicity and elaboration of other so-called "soluble mediators." A more fundamental understanding of the immunobiology of these crucial events and morphologic correlates may provide new insights into immune competance in man and information on the mechanisms of cellular regulation of the immune responsiveness. Potential approaches to therapeutic manipulation of such events would be of immediate clinical relevance. Indeed we have identified for the first time a specific in vitro site of action of corticosteroids in alteration of cellular immunity.

Proposed Course:

1. Characterize the mechanism of antigen recognition by immune T lymphocytes.

2. Characterize the ultrastructural features of guinea pig T lymphocytes.

3. Characterize the role of the macrophage in in vivo and in vitro initiation of cell-mediated immunity in experimental animals and man.

4. Characterize the localization and fate of antigen in in vitro antigen-mediated lymphocyte proliferation.

5. Explore the role of histocompatibility and genetic regulation in cell - cell interactions required for expression of cellular immunity.

Honors and Awards: NONE

Publications:

1. Rosenstreich, D. L., Shevach, E. M., Green, I., and Rosenthal, A. S.: The uropod-bearing lymphocyte of the guinea pig. Evidence for thymic origin. J. Exp. Med. 135: 1037-1048, 1972.

2. Rosenthal, A. S., Rosenstreich, D. L., Blake, J. T., Lipsky, P. E., and Waldron, J. A.: Mechanisms of antigen recognition by T lymphocytes. In Daguillard, F. (Ed.): Proceedings of the 7th Leukocyte Culture Conference. Quebec, Canada, Academic Press, Inc., 1972, pp. 201-216.

3. Rosenthal, A. S., Rosenstreich, D. L., Davie, J. M., and Blake, J. T.: Isolation and characterization of cellular immune effector cells: evidence for the heterogeneity os such cells in the guinea pig. In Proceedings of the Sixth Leucocyte Culture Conference. New York, New York, Academic Press, Inc., 1972, pp. 433-454.

4. Rosenstreich, D. L. and Rosenthal, A. S.: Peritoneal exudate lymphocyte. II. In vitro lymphocyte proliferation induced by brief exposure to antigen. J. Immunol. 110: 934-942, 1973.

5. Balow, J. E. and Rosenthal, A. S.: Glucocorticoid suppression of macrophage migration inhibitory factor. J. Exp. Med. 137: 1031-1041, 1973.

6. Waldron, J. A., Horn, R. G., and Rosenthal, A. S.: Antigen-induced proliferation of guinea pig lymphocytes in vitro: Obligatory role of macrophages in the recognition of antigen by immune T lymphocytes. J. Immunol. In press.

7. Rosenthal, A. S. and Rosenstreich, D. L.: The uropod: A specialized surface site for immunologic recognition. In Manson, L. (Ed.): Biomembranes. New York, Plenum Press. In press.

8. Cohen, B. E., Rosenthal, A. S., and Paul, W. E.: Antigen-macrophage interaction. I. Hapten-specific inhibition of antigen interaction with macrophages from immune animals. J. Immunol. In press.

9. Cohen, B. E., Rosenthal, A. S., and Paul, W. E.: Antigen-macrophage interaction. II. Relative roles of cytophilic antibody and other membrane sites. J. Immunol. In press.

Serial No. NIAID-47(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Clinical Studies of Complement Abnormalities of Man

Previous Serial Number: SAME

Principal Investigator: Dr. Michael M. Frank

Other Investigators: Dr. Joseph E. May
Dr. Robert Gerwin
Miss Thelma Gaither
Miss Marie Persinger

Cooperating Units: Dr. N. Hadler, A&R, NIAMD
Dr. J. Leddy, University of Rochester, Rochester, New York
Dr. W. Rosse, Duke University School of Medicine Durham, North Carolina

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 11/12 | | 7/12 | 1 - 6/12 |
| Professional: | 4/12 | | 7/12 | 11/12 |
| Other: | 7/12 | | -- | 7/12 |

Project Description:

Objectives:

To define the role of complement in human illness and to develop better methods of therapy for immune damage mediated by complement.

Methods Employed:

Complement components are purified and used for estimation of component levels in human sera and spinal fluid. Double blind therapeutic studies are employed to test new methods of therapy in patients. Patients on experimental drug protocols are followed to determine signs of toxicity. New methods are devised to diagnose complement related diseases and to investigate their pathophysiology.

Major Findings:

Epsilon amino caproic acid has been shown to be an effective agent in treatment of the disease Hereditary Angioneurotic Edema and the clinical toxicity of this drug has been defined in patients suffering with this disease. Complement component levels have been studied in several diseases; for example, study of the blood and spinal fluid from patients with systemic Lupus Erythematosis of the central nervous system has shown that C4 can be helpful in the diagnosis of this condition but it is not absolutely diagnostic. Repeated assays are required to establish the normal CNS C4 level for each patient and deviation from normal must be compared with each patient serving as his own control. The normal levels of C4 in spinal fluid and the stability of this component in spinal fluid stored in vitro have been evaluated as part of this study. Recent work has shown that it is possible to store CSF at -190°C with preservation of C4 stability.

The first patient with total C6 deficiency has been identified and a genetic analysis of C6 deficiency has been performed. This deficiency is inherited as a simple mendelian trait with heterozygous individuals having about half normal C6 levels. Availability of this patient has allowed for an evaluation of the role of human C6 in the clotting of human blood. C6 has been shown to play a distinctly minor role in the normal clotting reaction in contrast to what was shown in clotting studies in rabbits.

Preliminary studies indicate that lysis of erythrocytes from patients with the disease paroxysmal nocturnal hemoglobinuria proceeds in normal serum to which magnesium has been added. This had led to the development of a new diagnostic test for PNH and may lead to a new understanding of the pathophysiological basis of this illness.

Significance to Biomedical Research and the Program of the Institute:

These studies help define the role of complement in human illness and help to develop better diagnostic techniques for the detection of sick individuals. For example, better techniques have been made available for the detection of patients with paroxysmal nocturnal hemoglobinuria and for the diagnosis of Lupus Erythematosus involving the central nervous system. Medications are being developed to correct abnormalities of the complement system like those seen in Hereditary Angioneurotic Edema. The consequences of complement deficiency states are being defined by the discovery of patients with specific complement abnormalities as in the C6 deficient individuals that we have identified. Hopefully, an understanding of the role of complement in the production of disease will aid in our understanding of the prevention of these major illnesses.

Proposed course:

These studies will be extended to evaluation of new therapeutic agents. The exploration of the role of complement in clinical illness will be continued.

Honors and Awards:

Material from these studies was selected for presentation at meetings of the American Society for Clinical Investigation, and the American Rheumatism Association.

Publications:

1. Frank, M. M., Leddy, J. P., Gaither, T., Heusinkoeld, R. S., Breckenridge, R. T., and Klemperer, M. R.: Hereditary C6 deficiency in man. In Proceedings of the Second International Conference on Immunodeficiency Diseases in Man. In press.

2. Hadler, N. M., Gerwin, R. G., Frank, M. M., Whitaker, J. N., Baker, M., and Decker, J. L.: The fourth component of complement in the cerebrospinal fluid in systemic lupus erythematosus. Arthritis Rheum. In press.

Serial No. NIAID-48(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Antibody Responses in the Respiratory Tract of Rabbits Following Parenteral Immunization with Pseudomonas Aerginosa Vaccine and Enhancement of Bacterial Phagocytosis and Killing by Respiratory Macrophages with Immune Opsonic Antibodies

Previous Serial Number: SAME

Principal Investigator: Dr. Herbert Y. Reynolds

Other Investigators: Dr. James E. Pennington

Cooperating Units: Drs. Leo Orr and Arthur Levine, Infectious Disease Section, Hematology and Supportive Care Branch, NCI, NIH (Project No. 6830).
Drs. Robert Wood and Paul A. Di Sant-Agnese, Pediatric Metabolism Branch, NIAMD (Project No. 2c)

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 10/12 | | | 10/12 |
| Professional: | 7/12 | | | 7/12 |
| Other: | 3/12 | | | 3/12 |

Project Description:

Objectives:

Pseudomonas aeruginosa is a serious opportunistic bacterial pathogen for patients with impaired host immunity due to hematological malignancies or from immunosuppressive therapy for various neoplastic or other systemic diseases. Also, patients with extensive burns or structural lung diseases, particularly those who require assisted ventilation treatment, are at high risk to develop Pseudomonas infections. Pseudomonas colonization of the respiratory tract and subsequent pneumonia is particularly troublesome because the patient's response to antibiotics is often not dramatic and the microorganisms may not be eradicated. For example, pulmonary infections in children with cystic fibrosis are difficult to cure because antibiotics such as carbenicillin and gentamicin do not diffuse into bronchial secretions in sufficient concentrations to achieve inhibitory levels against the Pseudomonas organisms. Another approach to improve therapy of these pulmonary infections is that of immunological prophylaxis

through the development of specific anti-Pseudomonas antibodies in serum and in the respiratory tract. These antibodies, particularly those with opsonic antibody activity, may help the lungs' cellular defense mechanism (pulmonary macrophages) by enhancing phagocytosis and killing of Pseudomonas.

The initial phase was conducted in experimental animals and has expanded now into a more general assessment of pulmonary host defenses which includes several human experimental protocols, employing a heptavalent Pseudomonas aeruginosa vaccine (Parke-Davis, Inc. BB-IND 185), and in the study of an immunosuppressed canine model of experimental pneumonia. In many ways the clinical projects are consolidated in this experimental pneumonia model (Pseudomonas aeruginosa, serotype II), done in collaboration with Dr. David C. Dale (LCI) and Dr. Robert Graw (NCI).

Method Employed:

Rabbits are vaccinated with a variety of monovalent and polyvalent whole-cell or lipopolysaccharide Pseudomonas vaccines to observe the development of antibody in serum and bronchial secretions. Agglutinating antibodies, total titers and 2-mercaptoethanol resistant (IgG) titers, are measured. Isolation and purification of antibodies from serum and bronchial secretions employes gel filtration and ion exchange chromatography, sucrose density gradient ultracentrifugation, zone electrophoresis and other standard concentration and separation techniques. Macrophages, established in short-term cell monolayer cultures with well described techniques, are challenged with $^{14}C$ labeled bacteria, which have been previously opsonized; subsequent phagocytosis is measured with liquid scintillation spectroscopy and bacterial killing is assayed with standard agar pour plate cultures. Bronchial secretion proteins are quanitated by immunodiffusion precipitation methods. Human studies utilize many of the same methods.

Major Findings:

The type of specific anti-Pseudomonas antibody induced in the lungs or bronchial secretions depends upon the route of vaccination used - intramuscular (IM) or intranasal. Following parenteral (IM) immunization, IgG agglutinative and opsonic antibody is present in lung secretions which diffuses into the lung from the blood; in contrast, intranasal immunization produces secretory IgA and secretory IgG antibodies in addition to a modest systemic or serum antibody response. These bronchial antibodies have been isolated and characterized. However, IgG seems to be the more important opsonic antibody in the lung on the basis of phagocytic and killing studies done with in vitro alveolar macrophages and $^{14}C$ labeled Pseudomonas organisms.

Results of vaccination studies in three groups of susceptible patients to Pseudomonas infections, those with leukemia, cystic fibrosis, and structural lung diseases, are still preliminary but show that vaccination is tolerable in these patients, if small doses of steroids are combined in the syringe with the vaccine which effectively reduces systemic reactions to the lipopolysaccharide material. A good humoral antibody response can be obtained, and

Pseudomonas colonization and infection can be minimized. The vaccination studies were proceeded by a retrospective analysis of Pseudomonas pneumonia at the Clinical Center, NIH so that the most susceptible patient groups could be identified.

Significance to Biomedical Research and the Program of the Institute:

Pseudomonas infections in patients with altered immunity or with structural lung disease is a major infectious disease problem which causes a high number of acquired infections in hospitalized patients. Recognizing that patient isolation is not feasible because of the ubiquity of the Pseudomonas organisms, which are part of the bacterial flora of many hospitalized patients, and that antibiotic therapy may be ineffective despite the availability of good anti-Pseudomonas antibiotics, immunization of high risk patients with Pseduomonas vaccines is a reasonable addition to existing therapy. A Pseudomonas polyvalent vaccine, developed by Parke, Davis and Co., Detroit, Michigan, is currently under investigation (BB-IND 185).

As yet little attention has been given to the induction of protective antibodies in the respiratory tract and to the subsequent control of pulmonary colonization by Pseudomonas. Certainly, from the experimental animal studies described, it is possible to obtain specific bronchial antibody but to do so immunization must be prolonged enough to ensure development of IgG antibody. In the presence of the antibody which is available to opsonize Pseudomonas organisms, pulmonary macrophages in vitro are efficient in ingesting and killing these organisms.

Proposed Course:

1. Patients considered to be at high risk for Pseudomonas pulmonary infections, will be vaccinated with the Parke, Davis and Co., polyvalent Pseudomonas vaccine (Pseudogen®) to determine the optimal vaccination regimen necessary to produce specific IgG bronchial antibodies. Observations concerning the protective value of these antibodies will be made.

2. Continuation of the experimental pneumonia model in neutropenic and immunosuppressed dogs.

3. Studies with human respiratory macrophages and opsonic antibodies, similar to the animals experiments, will be continued.

Honors and Awards: NONE

Publications:

1. Reynolds, H. Y. and Thompson, R. E.: Pulmonary Host Defenses I. Analysis of protein and lipids in bronchial secretions and antibody responses following vaccination with Pseudomonas aeruginosa. J. Immunol. In press.

2. Reynolds, H. Y. and Thompson, R. E.: Pulmonary Host Defenses. II. Interaction of respiratory antibodies with Pseudomonas aeruginosa and alveolar macrophages. J. Immunol. In press.

3. Pennington, J. E., Reynolds, H. Y., and Carbone, P. P.: Pseudomonas pneumonia: A retrospective study of thirty-six cases. Amer. J. Med. In press.

Serial No. NIAID-49(c)
1. LCI
2. Clinical Parasitology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Laboratory Studies on Viruses of Entamoeba hystolytica and their Possible Significance in Human Amebiasis

Previous Serial Number: SAME

Principal Investigator: Dr. Peter T. Franck

Other Investigators: NONE

Cooperating Units: Dr. Louis S. Diamond, LPD, NIAID, NIH (Project no. 122-G)

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 6/12 | | | 6/12 |
| Professional: | 6/12 | | | 6/12 |
| Other: | -- | | | -- |

Project Description:

Objectives:

1) To investigate the biology of the virus, E. hystolytica host cell relationship, in axenic as well as bacteria-associated cultures.

2) To assess the prevalence of latent viruses in E. hystolytica strains obtained from patients with clinical amebiasis, in contrast to isolates from individuals with asymptomatic infection.

3) To investigate the role, if any, of viruses of E. hystolytica in the pathogenesis of human disease, either through direct effect on the human host or by alteration of the pathogenicity of E. hystolytica.

Methods Employed:

Strains of E. hystolytica are maintained in culture, either axenically or with a variety of bacterial associates. New isolates of E. hystolytica are sought, both from patients with disease and from symptomless carriers. The presence of virus is assayed by inoculating material from a new amebic isolate onto a battery of known ameba cell lines; conversely, new isolates are challenged with viruses known to be pathogenic for other amebic cell lines.

Major Findings:

All of the established E. hystolytica cell lines now in axenic culture originated from patients with severe clinical amebiasis, and all of these lines have been found by Dr. Diamond and co-workers to contain virus. An additional E. hystolytica isolate freshly obtained from a patient with amebic liver abscess and empyema was found to contain virus when tested after the fifth passage in culture with bacterial associates. Cell lines which had been maintained in axenic culture for long periods continued to produce virus when reassociated with bacteria and remained susceptible to exogenous virus. The study of isolates from asymptomatic carriers was hampered by difficulty in obtaining such isolates from patients who could be well studied clinically. Bacteria-associated E. hystolytica isolates from two symptomless individuals were examined, and no virus could be demonstrated with the techniques employed.

Significance to Biomedical Research and the Program of the Institute:

The wide variation in the apparent pathogenicity of E. hystolytica and the factors responsible for conversion of a symptomless carrier state to amebic disease remain unexplained and are of crucial significance to an understanding of the pathogenesis of amebiasis. Recognition of viruses of E. hystolytica warrants an investigation of their possible role in human disease.

Proposed Course:

This project is to terminate on June 30, 1973. Further investigation is to continue in Dr. Diamond's laboratory (under Project no. 122-G).

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-50(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mechanisms of Host Defense

Previous Serial Number: SAME

Principal Investigator: Dr. Joseph S. Bujak

Other Investigators: Dr. Michael J. Chusid
Dr. David C. Dale
Dr. John I. Gallin
Dr. Sheldon M. Wolff
Mr. George Ford

Cooperating Units: Dr. Parker Staples, University of Rochester, School of Medicine and Dentistry, Rochester, New York
Dr. W. Archie Bleyer, NCI, NIH
Mrs. Nettie Knight, NIAMD, NIH
Mr. Peter Kelsey, Undergraduate, Princeton University

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 1-5/12 | | | 1-5/12 |
| Professional: | 1-5/12 | | | 1-5/12 |
| Other: | -- | | | -- |

Project Description:

Selected patients studied in the laboratory included:

a. Patients with recurring bacterial infections including chronic granulomatous disease of childhood.

b. Patients with circulating rheumatoid factor.

c. Patients with hematologic malignancies.

d. Patients with eosinophilia.

Studies are in progress concerning the nature of defects in host defenses responsible for recurrent infections in these patients with particular attention paid to leukocyte function.

Objectives:

Human Studies:

1. To examine the adequacy of leukocyte function in patients with recurrent infections.

2. To investigate the mechanisms by which eosinophils kill bacteria.

3. To determine how rheumatoid factor may act to interfere with leukocyte phagocytosis of microorganisms.

4. To examine the functional capacity of leukemic leukocytes.

Animal Studies:

1. To investigate the phagocytic and bactericidal capabilities of beige mice to determine if they have the leukocyte abnormalities characteristic of the Chediak-Higashi syndrome.

2. To study the opsonic capacity of uremic rabbit serum to see if impaired phagocytosis contributes to the increased frequency of infections in uremia.

3. To study the peripheral blood neutrophils in grey collie dogs to see if an intrinsic abnormality is associated with their increased susceptibility to infections.

Methods Employed:

In vitro studies of host defense include chemotactic assays (Boyden chamber) and passive migration to study directed and random motility. Phagocytic function is assessed using $C^{14}$ labeled Staphylococcus aureus and thereby quantitating cell associated bacteria with time. Leukocyte bactericidal assays are performed using a pour plate technique. Metabolic functions are assessed by measurements of $O_2$ consumption ($O_2$ electrode), hexose monophosphate shunt ($C^{14}$-1 glucose oxidation) and Krebs cycle ($C^{14}$-6 glucose oxidation) activity, hydrogen peroxide formation ($C^{14}$-formate oxidation) and reduction of nitro-blue tetrazolium dyes. Iodination of intracellular protein with phagocytosis is determined by incubating leukocytes with $I^{125}$ and counting radioactivity in TCA precipitates of whole cells.

After dextran sedimentation, human blood eosinophils are purified using a hypaque gradient centrifugation technique and the metabolic, and bactericidal properties assessed.

Casein induced peritoneal exudates are harvested to provide leukocytes for the studies in C57 black mice and their beige variant.

Glycogen induced peritoneal exudates provide cells for the rabbit studies. Serum is obtained from normal New Zealand white rabbits and then the rabbits are made uremic by intramuscular injections of cephaloridine. Serum obtained from the uremic rabbits is compared to the same rabbits pre-uremic serum in the phagocytosis assay. Various serum chemistry determinations are obtained on those uremic sera with diminished opsonins in an effort to correlate any decrease in opsonization with a specific chemical change present in uremic serum.

Major Findings:

1. Investigations in a patient with candida sepsis secondary to perforated candida gastric abscesses revealed that all studies of phagocytic and bactericidal mechanisms relevant to bacterial killing were normal. However, killing of candida albicans was abnormal. This patient may have an abnormality in an as yet to be defined intraleukocytic microbicidal pathway that enables neutrophils to kill candida normally.

2. Eosinophils do not phagocytize bacteria as well as neutrophils and therefore are less effective in bactericidal assays. Eosinophils generate more $H_2O_2$ and contain more peroxidase than do neutrophils. Unlike neutrophils, however, eosinophil peroxidase plays no role in their killing mechanism(s). Sodium azide enhances eosinophil phagocytosis and thereby improves eosinophil bactericidal activity. The contribution of peroxidase to neutrophil killing of bacteria occurs only during the first 30 minutes following phagocytosis.

3. Serum of uremic rabbits is less effective in opsonizing S. aureus than pre-uremic control serum. This decrease in activity does not correlate with the alterations in blood urea nitrogen, creatinine, electrolytes, and glucose that occur with uremia. Uremic sera heated at 56°C for 30 minutes is still less efficient at opsonization than similarly treated pre-uremic controls. Mixtures of normal and uremic sera are intermediate in activity and therefore attempts to define an inhibitor or deficiency present in uremic serum have been unsuccessful.

4. Peritoneal exudate leukocytes from the beige variant of C57 black mice phagocytize but fail to kill S. aureus and group D streptococci as well as C57 black controls. Since these beige mice also have abnormal cellular chemotaxis, they share the abnormalities of leukocyte function known to occur in the Chediak-Higashi syndrome in man and thereby provide a laboratory model of this genetic abnormality in leukocyte function.

5. Thirty-seven percent of 40 sera with varying titers of rheumatoid factor activity are inhibitory to the opsonization of S. aureus. None of 8 normal sera contain this inhibitor. This inhibition is independent of the titer of rheumatoid factor and interferes with the opsonization of the microorganism rather than with leukocyte function.

6. Neutrophils from grey collie dogs do not kill S. aureus as well as controls. Phagocytosis is normal, but iodination of heat killed S. aureus is diminished despite having normal levels of myeloperoxidase. The exact nature of this intracellular defect remains to be elucidated.

Significance to Biomedical Research and the Program of the Institute:

These studies provide insight into the mechanisms which govern normal phagocytic and digestive activity of leukocytes and how they may be altered in various disease states. Such an understanding may conceivably lead to therapeutic correction of abnormalities and enhancement of normal activities when indicated.

Proposed Course:

This project will be incorporated into project 12(c) next year.

Honors and Awards: NONE

Publications:

1. Bujak, J. S., Aptekar, R. G., Decker, J. L., and Wolff, S. M.: Juvenile rheumatoid arthritis in the adult presenting as fever of unknown origin. Trans. Assoc. Am. Physicians LXXXV: 181-191, 1972.

2. Bujak, J. S., Aptekar, R. G., Decker, J. L., and Wolff, S. M.: Juvenile rheumatoid arthritis presenting in the adult as fever of unknown origin. Medicine In press.

3. Bujak, J. S., Ottessen, E. A., and Dinarello, C. A.: Nocardiosis in a child with chronic granulomatous disease. J. Pediatr. In press.

Serial No. NIAID-51(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Human Responses to Salmonella typhosa Vi Antigen (Clinical Research Project #73-I-01)

Previous Serial Number: NONE

Principal Investigator: Dr. Herbert Y. Reynolds

Other Investigators: Dr. Douglas M. Levin

Cooperating Units: (Co-principal investigator) Dr. Robert S. Northrup
Division of Bacterial Products, BB, NIH
Dr. K. H. Wong, Division of Bacterial Products, BB, NIH

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 1 | | | 1 |
| Professional: | 9/12 | | | 9/12 |
| Other: | 3/12 | | | 3/12 |

Project Description:

Objectives:

To test safety and immunogenicity of Salmonella typhosa Vi antigen in human volunteers. Typhoid vaccines in present use are quite reactive, producing local and systemic toxicity after injection, and do not provide complete protection against the disease. Typhoid vaccine trials comparing an acetone-inactivated dried vaccine with a heat- and phenol-killed vaccine demonstrated better protection with the acetone-treated preparation. This has been attributed to the destruction of Vi antigen by the heat-phenol process. Vi antigen prepared from citrobacter organisms shares antigenic determinants with S. typhosa Vi. Such a citrobacter Vi was used in human challenge studies. Protection was less than that seen with whole cell vaccine, but some protection occurred. For this reason, a Vi preparation from S. typhosa has been prepared (Vi-$Ty_2$) by a mild precipitation procedure. This preparation contains 3.5% endotoxin, and at a 50 µg human dose is 23-fold less toxic than a human dose of conventional vaccine, as tested in the actinomycin-D mouse endotoxin assay model. Protective potency in the mouse challenge model is approximately 16 times that of conventional vaccine, comparing them at doses relative to a human dose, and 176 times more potent than the Vi preparation used in the human challenge

studies. In addition, the Vi-$Ty_2$ is approximately three times more active in stimulating the in vitro thymidine uptake by mouse spleen lymphocytes than a citrobacter Vi preparation or than the conventional vaccine. Using the standard assays prescribed by Federal Regulations for licensed products, it is safe and non-toxic in guinea pigs and mice in toxicity assays, and is sterile.

Human studies to determine reactivity and immunogenicity in comparison with an acetone-killed and dried conventional typhoid vaccine (lot 6A, Bureau of Biologics reference standard) are underway. Data relative to the Vi-$Ty_2$ preparation was filed with the Bureau of Biologics for IND review prior to initiation of human studies. The vaccine preparation from which the 6A lots were derived has been used safely in humans in the past, and the 6A lots have passed standard safety and sterility tests.

Methods Employed:

Healthy normal subjects without history of previous typhoid immunization will be utilized. Blood samples (250-300 ml total for entire protocol) will be obtained for assay of antibody and cellular immunity prior to and at intervals following immunization over a 6-week period, and, where possible, 3-6 months later. Antigen will be injected intramuscularly following the drawing of a blood sample at 0 and 4 weeks. Observation of the volunteer will be carried out after each injection. This will include recording of vital signs four times daily for at least 3 days, and questioning of the patient for symptoms of systemic toxicity, including fatigue, anorexia, malaise, headache, and myaligia for at least 3 days.

Volunteers will be alternatively assigned to two groups. Five volunteers in the first group will receive 25 μg of Vi-$Ty_2$ in phospate buffered saline. Five volunteers in the second group will receive 0.5 ml of conventional acetone typhoid vaccine (type 6A Bureau of Biologics reference standard vaccine). At least one week later, a second five volunteers in the first group will receive 50 μg of Vi-$Ty_2$, and at least one week later a third group of five volunteers will receive 100 μg. A booster of their original dose will be given to all volunteers at 1 month if no untoward reactivity is noted.

All blood specimens will be analyzed for bactericidal and hemagglutinating antibody titers to S. typhosa. Lymphocytes will be separated from selected specimens and examined by MIF and thymidine uptake techniques for reactivity to typhoid antigens.

In addition to these studies, salivary and parotid secretions (Kirby cup) will be collected from selected volunteers at various times during the course of the study, and examined for secretory antibody. Also, intradermal injections of no more than three different dilutions of the Vi antigen (1 μg or less) or the whole cell vaccine will be performed 2 days prior to the initial immunization, and one week following the second injection, to assess early and delayed skin hypersensitivity. A skin test of the appropriate diluent will be done concomitantly, for comparative purposes.

Major Findings:

Preliminary results only are available. The first group of volunteers were inoculated in February, 1973.

Significance to Biomedical Research and the Program of the Institute:

Hopefully, an improved vaccine to protect against typhoid fever will be found.

As the Vi-$Ty_2$ preparation is substantially reduced in toxicity from conventional typhoid vaccine, markedly reduced local and systemic reactions can be expected. Animal work would indicate that this new vaccine has good immunogenicity so that antibody response and possibly cellular immunity will be good.

Proposed Course:

1. Following preliminary immunization of small groups with 25 $\mu$g, 50 $\mu$g and 100 $\mu$g doses of Vi-$Ty_2$, two additional groups of five volunteers will be given Vi-$Ty_2$ at a dose to be selected on the basis of information derived from the above studies, for confirmation of the data. An additional group of five may also be given typhoid vaccine if the data from the first five are inconclusive.

2. Field trials with the vaccine may be undertaken, pending success with the initial immunization studies.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID 52(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Antibiotic Kinetics in Respiratory Secretions

Previous Serial Number: NONE

Principal Investigator: Dr. Herbert Y. Reynolds

Other Investigators: Dr. James E. Pennington

Cooperating Units: NONE

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 1 | | | 1 |
| Professional: | 9/12 | | | 9/12 |
| Other: | 3/12 | | | 3/12 |

Project Description:

Objectives:

A variety of gram negative bacteria are pathogens for patients with impaired host immunity due to hematological malignancies or immunosuppressive therapy for various neoplastic or other systemic diseases. Pseudomonas aeruginosa colonization of the respiratory tract and subsequent pneumonia is particularly troublesome because the patient's response to antibiotics often is not dramatic and the microorganisms may not be eradicated. For example, pulmonary infections in children with cystic fibrosis are difficult to cure because antibiotics such as carbenicillin and gentamicin do not diffuse into bronchial secretions in sufficient concentrations to achieve inhibitory levels against the Pseudomonas organisms (Marks et al, J. Pediat. 74: 822, 1971). Therefore, within the scope of a larger project to investigate pulmonary host defenses (Serial #NIAID-48(c)), we were interested to study the penetration of antibiotics into the lung and respiratory secretions and to observe the clearance of antibiotics from these secretions. An animal model was designed to study the kinetics of antibiotics in normal respiratory secretions. These studies are to be extended to patients where possible. Initially, antibiotics used specifically for Pseudomonas infections were evaluated.

Methods Employed:

Anesthetized and intubated dogs are used; blood (serum) and bronchial secretion samples are simultaneously obtained following administration of a variety of antibiotics which has included: penicillin G, ampicillin, getamicin, carbenicillin, tobramycin and 5-fluorocytosine. Antibiotic concentrations are measured using a cup plate agar method (Bennett et al., Applied Microbiol. 14: 170, 1966). Bronchial secretions are also analyzed for protein content and quanitatively for albumin and various classes of immunoglobulins (Reynolds and Johnson, J. Immunol. 105: 698, 1970).

Major Findings:

Following injection of maximal doses of gentamicin or carbenicillin, levels in normal bronchial secretions were obtained that would inhibit about 63% and 43%, respectively, of 1500 Pseudomonas aeruginosa isolates for which the mean inhibitory concentrations (MIC) were known. Tobramycin concentrations in bronchial fluids were equal to the MIC's reported for about 70% of Pseudomonas isolates. In contrast, penicillin G, which has demonstrated efficacy for such lung infections as pneumococcal pneumonia, was found to achieve levels 40 X the MIC's of over 99% of pneumococci isolated at NIH. The particulars of the canine model were also detailed in hopes that this might become an acceptable in vivo model for studying respiratory antibiotic clearances. Of note is that clearance of antibiotics from the lungs is appreciable slower than from serum which raises possibilities for increasing the effective concentration of antibiotics in the respiratory tract.

Significance to Biomedical Research and the Program of the Institute:

Antibiotic treatment for certain kinds of bacterial pneumonia produces variable rates of cure and often seems to be ineffective in specific infections such as Pseudomonas pneumonia where the mortality rate in most clinical series approaches 80%. A factor of importance is that antibiotics as currently administered may not reach sufficient concentrations in the lung or bronchial secretions to inhibit growth of the infecting micro-organisms. Methods to increase the effective level of antibiotics in the bronchial tract, such as direct instillation by aerosol or combinations of parenteral and aerosol administration, should be investigated.

Information about the clearance of antibiotics from the respiratory secretions is sparse compared with similar data about renal or hepatic clearance and excretion. The dog model used in this project may be useful for these studies.

Proposed Course:

1. Additional antibiotics will be examined in the dog model such as those used in the treatment of chronic bronchitis (Ampicillin, tetracycline and trimethoprin and sulfa) and fungal disease (5-FC).

2. Anti-Pseudomonas antibiotics will be measured in respiratory se-secretions of immunosuppressed dogs with experimentally induced Pseudomonas pneumonia.

3. Confirmation of antibiotic clearance in respiratory secretions will be attempted in suitable patients with tracheostomies.

Honors and Awards: NONE

Publications:

1. Pennington, J. E. and Reynolds, H. Y.: Gentamicin and carbenicillin concentrations in bronchial secretions. J. Infect. Dis. In press.

2. Pennington, J. E. and Reynolds, H. Y.: Tobramycin in bronchial secretions. Antimicrob. Agents Chemother. In press.

Serial No. NIAID-53(c)
1. LCI
2. Medical Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Etiology and Clinical Characteristics of Acute Infectious Nonbacterial Gastroenteritis

Previous Serial Number: NONE

Principal Investigators: Dr. Raphael Dolin
Dr. Julius A. Kasel (until 11/1/72)

Cooperating Units: Dr. Robert M. Chanock, LID, NIAID, NIH (no project no.)
Dr. Albert Z. Kapikian, LID, NIAID, NIH (Project no. 66H)
Dr. Richard G. Wyatt, LID, NIAID, NIH (Project no. 66H)

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 1-1/12 | | | 1-1/12 |
| Professional: | 1-1/12 | | | 1-1/12 |
| Other: | -- | | | -- |

Project Description:

Objectives:

1) To define the biologic, immunologic, and epidemiologic properties of the etiologic agents (viruses) of acute infectious nonbacterial gastroenteritis.

2) To develop in vitro culture systems and/or laboratory animal models with which to study these agents.

3) To examine the host response to infection with these agents, by the experimental induction of disease in normal volunteers.

Methods Employed:

1) Volunteer studies. Seven viral agents have been obtained from naturally occurring outgreaks of acute infectious nonbacterial gastroenteritis (AINBG) throughout the United States. After rigorous safety testing in culture and animal systems, these agents were administered and serially passed in volunteers to establish the infectivity of the agents and to provide suitable clinical specimens for further study.

2) Cultivation and characterization of the agents. The infectious material obtained from naturally occurring and experimentally induced disease was inoculated into standard tissue culture systems, laboratory animals, and the recently developed human fetal intestinal organ cultures. Particular emphasis was placed on the organ cultures, since they represent the only in vitro system in which replication of these agents has been detected. The cultures were examined for direct and indirect evidence of viral growth, employing light and electron microscopy, viral interference, and metabolic changes within the cultures. Acute and convalescent sera from volunteers and naturally occurring cases were employed in immunofluorescence and immunoaggregation techniques.

3) Host response in man. In addition to blood samples, small intestinal and colonic secretions were obtained to search for local as well as systemic antibody production. The role of cell-mediated immunity was studied by establishing lymphocyte cell cultures from the volunteers. Peroral intestinal biopsies were examined for histopathology and in vitro enzyme and immunoglobulin production. The effects of the acute viral infection on the absorptive, secretory, and motility properties of the gastrointestinal tract were also studied.

Major Findings:

1) Further characterization of Norwalk agent. Prior studies indicated that the Norwalk agent of AINBG was less than 36 nm in diameter, lacked a lipid coat, was resistant to acid treatment (pH 2.7), and was resistant to heating at 60°C for 30 minutes. Utilizing electron microscopic immuno-aggregation techniques, a 27 nm particle with properties consistent with those described above, was detected in infectious stool filtrates (Kapikian, Wyatt, Dolin). Convalescent sera from four volunteers in whom disease was induced by administration of these filtrates demonstrated a rise in serum aggregating properties. Similar rises were seen in sera from three of five naturally occurring cases in the Norwalk outbreak.

2) Host response in infection with Norwalk agent. Peroral, small intestinal biopsies of four ill volunteers demonstrated light and electron microscopic changes consisting of blunting of villi, denudation of brush border, vacuolization of cytoplasm, and intercellular deposits of electron-dense material. The biopsies during acute illness also showed lowered values of the intestinal enzymes, sucrase, alkaline phosphatase, and trehalose. Two weeks after acute illness, histology and enzyme levels had returned to normal. Three volunteers who remained well after administration of the Norwalk agent did not show the above abnormalities in their biopsies. In the volunteers who became ill, in vitro IgA production by explanted jejunal biopsies was elevated in biopsies taken two weeks after illness; while in the volunteers who remained well, IgA synthesis was elevated in the biopsy taken 72 hours after inoculation. These findings are consistent with possible local immunity to the Norwalk agent.

Significance to Biomedical Research and the Program of the Institute:

Acute infectious nonbacterial gastroenteritis is a major infectious disease of uncertain etiology which affects a large portion of the population in the United States and throughout the world. Identification of its etiologic agents

and an understanding of its pathogenesis are initial steps toward the ultimate goals of prevention and therapy. Further studies in volunteers, along with laboratory investigations, should continue to provide useful information concerning the causative agents and clinical course of this disease.

Proposed Course:

Intensive study of the etiologic agents of AINBG will continue, employing both laboratory techniques and volunteer studies. The biologic, immunologic, and epidemiologic properties of the agents will be further defined, along with any possible interrelationships among them. The host response to experimentally induced infection in man will also be investigated, with particular emphasis on the roles of local and systemic antibodies, non-specific defense mechanisms, and cellular immunity.

Honors and Awards: NONE

Publications:

1. Kapikian, A. Z., Wyatt, R. G., Dolin, R., Thornhill, T. S., Kalica, A. R., and Chanock, R. M.: Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. J. Virol. 10: 1075-1085, 1972.

2. Agus, S., Dolin, R., Wyatt, R. G., Tousimis, A. S., and Northrup, R. S.: Acute infectious nonbacterial gastroenteritis: Intestinal histopathology. Ann. Intern. Med. In press.

Serial No. NIAID-54(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Regulation of the Immune Response in Man and Experimental Animals

Previous Serial Number: NONE

Principal Investigator: Dr. Anthony S. Fauci

Other Investigators: Dr. Douglas L. Hurley
Dr. Sheldon M. Wolff
Dr. David C. Dale
Miss Kathy O'Meara

Cooperating Units: NONE

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 3 | | | 3 |
| Professional | 1-4/12 | | | 1-4/12 |
| Other: | 1-8/12 | | | 1-8/12 |

Objectives:

1) To delineate the precise mechanisms whereby immunosuppressive agents such as cytotoxic drugs and corticosteroids affect the immune response in man and in experimental animals, specifically looking at in vivo and in vitro function of the monocyte and subpopulations of lymphocytes.

2) To investigate at the cellular and subcellular levels various states of altered immune responsiveness in situations other than those described in objective #1. This includes hypersensitivity states, granuloma formation, and autoimmune phenomena which occur in disease states in man, and which are artificially produced in animals.

3) To study the mechanisms whereby altered states of immunity as described above affect host mechanisms against infections.

4) To clinically evaluate patients with prolonged fever of unknown etiology and to investigate new possible mechanisms for their disorders.

Methods Employed:

1) A significant proportion of the methodology is centered around the in vitro culturing of lymphoid cells to determine their capability of reacting to certain stimuli. The effects of in vivo chemotherapeutic agents as well as the effects of disease-related altered states of immunity can thus be monitored and explored.

2) Administering corticosteroids to normal volunteers and patients, and measuring the precise cellular effects on the immune response.

3) Labeling a subject's lymphoid cells with $^{51}$Chromium and reinfusing the cells back into their circulation to determine the effect of disease states and of chemotherapeutic agents on circulation patterns and viability of these cells.

4) Identifying subpopulations of lymphoid cells by their surface markers and determining the change in relative proportions of such cells in disease states and during chemotherapy.

5) Producing altered states of immunity in experimental animals by x-irradiation, surgical removal of lymphoid organs, or administration of cytotoxic agents in order to determine precisely the requirement of certain factors in the immune response necessary for host defense and immunological surveillance.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

1) A group of patients with Wegener's granulomatosis (a disease characterized by granulomatous vasculitis involving the respiratory tract and kidneys) are being studied. Untreated, the disease is rapidly fatal. We are currently following the largest single group of such patients in the world. The disease serves as an excellent model of a hypersensitivity state. In addition to effecting long term remissions and even cures in this formerly fatal disease by the use of cytotoxic agents (Cyclophosphamide), we were able to clearly demonstrate that on our therapeutic protocol there was suppression of particular aspects of the immune response (immunoglobulin levels, newly induced delayed hypersensitivity) with sparing of other aspects (established delayed hypersensitivity, in vitro blastogenic response to mitogens, and relative proportions of subpopulations of lymphocytes). This degree of immunosuppression was correlated with clinical remission of disease and was not associated with an increased incidence of infection.

2) Corticosteroids were administered to normal volunteers in order to determine the precise effects upon the immune response. These agents were found: a) to cause a profound but transient decrease in lymphocyte and monocyte counts, b) to cause a change in the relative proportion of lymphocyte subpopulations in the circulation, and c) to cause a differential

and selective depression of certain lymphoid cell functions as measured by in vitro stimulation. This is the first time that such effects have been clearly demonstrated in humans.

Proposed Course:

The above studies will be expanded and examined in more detail both in disease states, normal controls, and in experimental animals.

Honors and Awards: NONE

Publications:

1. Northrup, R. S. and Fauci, A. S.: Adjuvant effect of cholera enterotoxin on the immune response of the mouse to sheep red blood cells. J. Infect. Dis. 125: 672-673, 1972.

2. Fauci, A. S. and Wolff, S. M.: Wegener's granulomatosis: Studies in eighteen patients and a review of the literature. Medicine In press.

3. Fauci, A. S. and Wolff, S. M.: Wegener's granulomatosis. In Schreiner, G. E. (Ed.): Perspectives in Nephrology. New York, John Wiley and Sons In press.

Serial No. NIAID-55(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Granulocyte Physiology

Previous Serial Number: NONE

Principal Investigator: Dr. David C. Dale

Other Investigators: Dr. Joseph S. Bujak
Dr. Michael J. Chusid
Dr. Charles A. Dinarello
Dr. Ronald J. Elin
Dr. Anthony S. Fauci
Dr. DuPont Guerry
Dr. James E. Pennington
Dr. Herbert Y. Reynolds
Dr. Sheldon M. Wolff
Mr. Stanley B. Ward
Mr. Edward W. Harvey
Mrs. Rhoda T. Hubert

Cooperating Units: Dr. John Adamson, University of Washington, Department of Medicine, Seattle, Washington
Dr. David W. Alling, OSD, NIAID, NIH (No project no.)
Dr. Jeffery S. Borer, NHLI, NIH (No project no.)
Dr. Robert Graw, NCI, NIH (Project no. 6820)
Dr. Mitsuhiro Omine, NCI, NIH (No project no.)
Dr. Seymour Perry, NCI, NIH (No project no.)
Dr. Howard Hoffman, NICHHD, NIH (No project no.)

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 4-4/12 | | | 4-4/12 |
| Professional: | 2-4/12 | | | 2-4/12 |
| Other: | 2 | | | 2 |

Project Description:

Patients and animal studies are conducted to investigate the regulation of granulocytopoiesis and the role of granulocytes in inflammation and defense against infection.

Objectives:

Human Studies:

1. To study the factors regulating the blood granulocyte count of normal individuals and patients with primarily infections, inflammatory, immunologic, and hematologic diseases.

2. To determine the pathophysiologic mechanisms of chronic and cyclic neutropenia.

3. To investigate the mechanisms of the hypereosinophilic syndrome. To study the role of eosinophils in causing the peculiar tissue injury seen in this disorder.

4. To assess the effects of immunosuppressive agents on granulocyte physiology. To better define the factors of increased host susceptibility to infection caused by these agents and better understand the basic mechanisms of immunosuppression.

5. To clinically evaluate patients with prolonged unexplained fever to delineate new inflammatory diseases and host defense abnormalities.

Animal Studies:

1. To define the mechanisms of cyclic hematopoiesis in grey collie dogs. To explore in these animals methods for altering cyclic neutropenia which may be applicable to the similar human disorder.

2. To study the treatment of experimental pneumonia in leukopenic dogs. To evaluate the relationship of the blood granulocyte count to susceptibility to infection and the use of granulocyte transfusion therapy.

Methods Employed:

Granulocyte counts are measured for long periods to diagnose and investigate cyclic neutropenia and other disorders of neutrophil production.

The blood granulocyte count is measured after administration of etiocholanolone, endotoxin, cortisol and prednisone to evaluate the bone marrow granulocyte reserve.

The blood granulocyte pool sizes, distribution and turnover rates are measured by radioisotopic labeling studies employing $DF^{32}P$ and $^{51}Cr$.

In vivo studies of leukocyte chemotaxis are measured by the skin window technique.

The kinetics of bone marrow cell formation are examined in vivo and in vitro with tritiated thymidine cell labeling, liquid scintillation counting and radioautography.

Factors effecting the proliferation of bone marrow cells are measured in vitro by the culture of bone marrow cells in a semi solid culture medium.

Pseudomonas pneumonia is induced in lethally irradiated dogs by direct endobronchial instillation of bacteria. The course of the infection is evaluated by serial x-rays, blood culture, blood counts, temperature measurements, and limulus assays for endotoxemia. Leukocytes procured from dogs by leukophoresis are administered daily.

Patient Material:

Selected patients are hospitalized in the Clinical Center for these studies.

Major Findings:

1. Cyclic neutropenia in dogs and man is a disorder of hematopoietic cell production probably due to a defect in stem cell regulation.

2. Chronic neutropenia is a benign disorder in most patients even with blood granulocyte counts below $500/mm^3$. The marrow reserve responses of these patients are proportional to the blood counts. Most patients appear to have defective or ineffective neutrophil production. Two patients with repeated life threatening infections have been found to improve remarkably with chronic alternate day steroid treatment.

3. Patients with malaria have neutropenia due to a redistribution of cells from the marrow reserve into an enlarged marginal pool.

4. The blood neutrophil counts in normal individuals do not show cyclic fluctuations as had been previously suggested.

5. Effective immunosuppression can be achieved in Wegener's granulomatosis without neutropenia or a depression of the acute inflammatory response.

Significance to Biomedical Research and the Program of the Institute:

These studies directly relate to understanding the role of granulocytes in resistance to infection.

Proposed Course:

The studies outlined will be continued. Studies of granulocyte function will be examined in greater detail. Studies of the marrow regulation of granulocyte production will be extended.

Honors and Awards: NONE

Publications:

1. Dale, D. C. and Wolff, S. M.: Cyclic neutropenia in man and grey collie dogs. In Bergsma, D. (Ed.) Birth Defects: Original Article Series. The Fourth Conference on the Clinical Delineation of Birth Defects. Part XIV: Blood. Baltimore, The Williams and Wilkins Co., Vol. 8, 1972, pp. 59-62.

2. Dale, D. C., Ward, S. B., Kimball, H. R., and Wolff, S. M.: Studies of neutrophil production and turnover in grey collie dogs with cyclic neutropenia. J. Clin. Invest. 51: 2190-2196, 1972.

3. Dale, D. C., Alling, D. W., and Wolff, S. M.: Cyclic hematopoiesis: The mechanism of cyclic neutropenia in grey collie dogs. J. Clin. Invest. 51: 2197-2204, 1972.

4. Dale, D. C., Alling, D. W., and Wolff, S. M.: Application of time series analysis to serial blood neutrophil counts in normal individuals and patients receiving cyclophosphamide. Br. J. Hematol. 24: 57-64, 1973.

5. Dale, D. C. and Wolff, S. M.: Studies of the neutropenia of acute malaria. Blood. 41: 197-206, 1973.

6. Scott, R. E., Dale, D. C., Rosenthal, A. S., and Wolff, S. M.: Cyclic neutropenia in grey collie dogs. Ultrastructural evidence for abnormal neutrophil granulopoiesis. Lab. Invest. 28: 514-525, 1973.

7. Dale, D. C. and Petersdorf, R. G.: Corticosteroids and infectious diseases. Med. Clin. N. Am. In press.

8. Geelhoed, G. W., Kane, M. A., Dale, D. C., and Wells, S. A.: Colonic perforation in cyclic neutropenia: Report of a survivor with peritonitis and clostridial septicemia. J. Pediatr. Surg. In press.

9. Dale, D. C., Fauci, A. S., and Wolff, S. M.: The effect of cyclophosphamide on leukocyte kinetics and susceptibility to infection in patients with Wegener's granulomatosis. Arthritic Rheum. In press.

Serial No. NIAID-56(c)
1. LCI
2. Allergic Diseases Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Biochemical Pathways of Mediator Release and Mechanism of Tissue Injury in Allergic Diseases

Previous Serial Number: NONE

Principal Investigator: Dr. Allen P. Kaplan

Other Investigators: Dr. John I. Gallin
Dr. Louis W. Heck
Dr. Charles H. Kirkpatrick
Dr. Eric A. Otteson
Dr. Alan S. Rosenthal
Mr. Henry Meier
Mr. Allen Weiss

Cooperating Units: Dr. Fletcher Taylor, Head Allergic Diseases Section, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 3-1/12 | - | - | 3-1/12 |
| Professional: | 1-9/12 | - | - | 1-9/12 |
| Other: | 1-4/12 | - | - | 1-4/12 |

Project Description:

Objectives:

1. Determination of the interrelationship of the coagulation, fibrinolytic, kinin-generating, and complement pathways in human plasma. Attention is focused upon the mechanism of generation of peptides which possess kinin-like activity and the role of anaphylatoxins in the liberation of histamine from tissue mast cells. The mechanism by which a kinin is derived from the complement pathway (C-kinin) is being investigated.

2. Development of radioimmunoassays or radial immunodiffusion assays for the assessment of the bradykinin forming pathway in human plasma. This will necessitate the development of a quantitative assay for Hageman factor and prekallikrein, the proenzymes required for the generation of bradykinin

as well as for the substrate kininogen and the nonapeptide, bradykinin.

3. Development of specific assays for the determination of histamine and slow reacting substance of anaphylaxis in human plasma as well as in other body fluids.

4. Investigation of mechanism of interaction of the chemotactic enzymes kallikrein and plasminogen activator with the surface of the neutrophil in order to initiate chemotaxis.

5. To identify the chemotactic factors which are generated by activation of the Hageman factor dependent pathways (coagulation, fibrinolysis, and kinin generation) in human serum or plasma.

6. Study of the significance of the liberation of a Platelet Activating Factor and its attendant seratonin release as a consequence of IgE-dependent release of mediators from human basophils.

7. Investigation of the role of thymic-dependent lymphocytes in order to initiate IgE synthesis and to subsequently cause IgE synthesis to cease (thymic suppressor cells). An abnormality in the latter function may be responsible for the heightened IgE response characteristic of "atopic diseases" such as hay fever and extrinsic asthma. Any soluble mediators released from T-cells which play a role in IgE synthesis or suppression will be isolated and characterized.

Methods Employed:

Purification of plasma proteins is performed utilizing affinity chromatography, ion exchange chromatography on Quarternary aminoethyl (QAE) Sephadex and Sulphopropyl (SP) Sephadex, gel filtration, and preparative elution from gels after isoelectric focusing. These methods have been applied to the isolation of unactivated Hageman factor, Hageman factor prealbumin fragments, pre-PTA (coagulation factor x 1), prekallikrein, plasminogen proactivator, plasminogen and the plasma inhibitors $\alpha_1$ antitrypsin, $\alpha_2$ macroglobulin, and the inhibitor of the activated first component of complement ($\overline{C1}$ INH).

The chemotactic activity generated by kaolin activation of human serum or plasma is assessed utilizing the radioassay described by Gallin et al (J. Immunol. 110: 233, 1973).

Major Findings:

1. "Fletcher factor" deficient plasma possesses a diminished rate of activation of the intrinsic coagulation pathway in the absence of a deficiency of any known coagulation factor. Contact activation of Hageman factor in this plasma has been shown to yield no bradykinin because it is deficient in prekallikrein. We have described a defect in kaolin activated fibrinolytic activity and in kaolin activated chemotactic activity in this plasma.

The coagulation, fibrinolytic, and chemotactic defects can be corrected by prolonged incubation with kaolin or by reconstitution with either prekallikrein or activated Hageman factor. The kinin-generating defect is corrected only by reconstitution with prekallikrein. Furthermore the product of Hageman factor activation of prekallikrein, i.e., kallikrein, activates unactivated Hageman factor. Thus a feedback mechanism exists in which kallikrein activates Hageman factor without which the three Hageman factor dependent pathways proceed at a markedly diminished rate. The results indicate that the activation of Hageman factor which leads to coagulation, fibrinolysis, and kinin generation requires not only contact activation to initiate the process but also fluid phase activation of Hageman factor.

The determination of kaolin activatable chemotactic activity in normal serum represents the first demonstration of a chemotactic pathway in whole serum which is independent of complement. Two plasmas possessing defects in kaolin-activatable chemotactic activity are Hageman factor deficient plasma and Fletcher factor (prekallikrein) deficient plasma.

Significance to Biomedical Research and the Program of the Institute:

This program represents the beginning of a commitment to investigate allergic diseases in the clinical center. The interrelated enzymatic pathways consisting of coagulation, fibrinolysis, kinin-generation, and complement represent one aspect of the inflammatory response which is being evaluated in patients with various types of chronic angioedema and chronic urticaria. The primary focus is upon the idiopathic forms which are the most common but in which the vasoactive mediators are unknown. Other types of chronic urticaria and angioedema being studied include hereditary angioedema, cold urticaria, cholinergic urticaria, and urticarial erythema multiforme. In many of these entities, and in hereditary angioedema in particular, activation of all of the above pathways appears to occur in the fluid phase in the absence of circulating immune complexes. The observed abnormalities present in Fletcher factor deficient plasma emphasize the importance of fluid phase activation of Hageman factor and changes our concept that this is necessarily a surface or contact-activated system. The generation of chemotactic activity as a result of activation of the Hageman factor dependent pathways provides a further link between the coagulation, fibrinolytic, and kinin-generating pathways and the recruitment of neutrophils into an area of inflammation. Identification of the vasoactive mediators liberated in this diverse group of chronic urticarias and angioedemas and further characterization of the enzymatic interactions which lead to their elaboration and destruction should lead to an understanding of the pathophysiology of these diseases and hopefully to new modes of therapy.

Proposed Course:

To continue the studies outlined above, develop the techniques needed to evaluate all of the mediator systems in patients with urticaria and angioedema and then initiate studies of intrinsic and extrinsic asthma.

Honors and Awards: NONE

Publications: NONE

INDEX

LABORATORY OF INFECTIOUS DISEASES


| | | Page |
|---|---|---|
| Summary Statement | | 1 |
| 66A | Epidemiologic Studies of Infections and Illness, and Their Control in Infants and Children | 11 |
| 66H | The Etiology of Acute Infectious Nonbacterial Gastroenteritis | 14 |
| 66I | The Natural History and Defective Nature of Infection with Adeno-Associated Viruses (AAV) | 25 |
| 69A | Study of Nonbacterial Respiratory Disease A. Children | 26 |
| 69C | The Etiology of Acute Respiratory Diseases in Adults | 33 |
| 70A | Laboratory Studies of Myxoviruses, Paramyxoviruses and Respiratory Syncytial Virus | 39 |
| 70B | Classification and Characterization of Rhinovirus Serotypes of Human Origin | 52 |
| 70C | Studies on Mycoplasmas | 56 |
| 70D | Laboratory and Epidemiologic Studies of Viral Hepatitis Agents. | 63 |
| 70E | Biological and Biophysical Studies of Rhinoviruses | 74 |
| 72A | New Techniques for Recovery of Respiratory Tract Viruses. | 75 |
| 72B | Study of Respiratory Viruses and Mycoplasmas in Human Volunteers | 83 |
| 72C | Role of Delayed Hypersensitivity in the Immune Response to Respiratory Viral Infections | 90 |

SUMMARY STATEMENT - Annual Report of the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases
July 1, 1972 through June 30, 1973

The research programs of LID are concerned with the natural history and prevention of three of the major uncontrolled infectious diseases of man - acute respiratory tract disease, viral hepatitis and acute gastroenteritis. Specifically, efforts are made to recover the microbial agents responsible for these diseases and to characterize the etiologic viruses or mycoplasmas in the laboratory. After techniques for the isolation of the etiologic agents and measurement of specific antibody have been developed, an attempt is made to study the natural history of infection, i.e., when and where does infection occur, what is the risk of disease during infection, and which environmental and host factors influence infection and expression of disease. Special emphasis is given to defining the immunologic mechanisms by which the host protects himself from infection and disease. Finally, information which emerges from the foregoing studies is used to devise a strategy for prevention of illness.

Acute Non-bacterial Gastroenteritis. This represents the newest program in LID. Work was begun in this area 3 years ago because of the public health importance of the disease syndrome — acute non-bacterial gastroenteritis is the second most common disease experience of contemporary man in the U.S. — and because a number of new techniques for recovery or recognition of microbial agents had been developed since this syndrome was last investigated in any detail.

We were successful in transmitting the self-limited disease of acute non-bacterial gastroenteritis to volunteers using as inoculum stool filtrates derived from 7 separate outbreaks. In each instance the stool filtrate was free of detectable bacteria. Each of the filtrates produced a spectrum of symptoms, including vomiting and/or diarrhea and/or abdominal pain, which was indistinguishable from the naturally occurring disease. Further characterization of one of the agents (Norwalk agent) in volunteers indicated that the agent was small, i.e., less than 66 nm and probably less than 36 nm in diameter, and not inactivated by ether, acid pH or incubation at 60°C for 30 minutes. After numerous efforts to grow the Norwalk agent in conventional tissue cultures failed we attempted to visualize the agent by electron microscopy. Stool filtrates from infected volunteers were examined by immune electron microscopy using inactivated convalescent serum from ill volunteers as the source of antibody. This approach was successful and we were able to visualize a 27 nm diameter "virus-like" particle in stool filtrates from ill volunteers. Furthermore, it was possible to demonstrate a serologic response to the 27 nm particle following both natural and experimental Norwalk gastroenteritis. This finding suggested that the 27 nm "virus-like" particle was indeed the etiologic agent of Norwalk gastroenteritis.

Using immune electron microscopy and standard isopycnic banding procedures the density of the 27 nm particle was shown to be 1.38-1.41 gm/cm$^3$ in cesium chloride. Although the type of nucleic acid present in the particle has not been established as yet, the morphology of the particle, its 1.38-1.41 density and resistance of the agent to 60°C for 30 minutes suggests that the Norwalk

agent is a parvovirus. If this supposition is confirmed the Norwalk agent will be the first parvovirus shown to cause disease in man.

During the past 3 years considerable effort was directed toward developing a human fetal intestinal organ culture system for growth of the fastidious agents of acute non-bacterial gastroenteritis. Recently evidence was obtained during a collaborative study with Dr. Neil Blacklow of the Boston University School of Medicine that one of the agents of acute gastroenteritis grew in this type of culture. A disease-producing stool filtrate derived from an outbreak in Hawaii was passaged 11 times serially in human fetal intestinal organ culture and the 11th passage culture harvest, representing a $10^{-13}$ dilution of the original inoculum,produced typical gastroenteritis in 3 of 14 volunteers.

Currently efforts are underway to detect "virus-like" particles in stool filtrates derived from outbreaks other than Norwalk and to grow these agents in human fetal intestinal organ culture and monolayer tissue culture. Finally, the relationship of agents from different outbreaks is being investigated by cross challenge in volunteers (Kapikian, Wyatt, Thornhill, and Kalica).

Hepatitis. The ultimate objective of the hepatitis program of LID, is to develop methods for the control of viral hepatitis, including, where applicable, the use of passive or active immunization and the interdiction of spread of the disease through identification of carriers or vectors of infection. The approaches we have taken include (a) the collection of clinical material of high potential infectivity for use in laboratory studies; (b) the development of new in vitro procedures and animal systems for use in laboratory studies of viral hepatitis; (c) an attempt to propagate the viruses of hepatitis A and B in culture or in experimental animals; and (d) study of the biochemistry, biophysics, immunology and epidemiology of hepatitis associated antigen.

Considerable effort has been expended on the study of the various morphological forms of hepatitis B antigen, one of which is believed to be the hepatitis B virus (HBV). Sensitive assays for the study of these antigens were developed; these included a modified radioimmunoassay for the detection of minute quantities of hepatitis B antigen (HB Ag) and a radioimmunoassay for the detection of the internal component (probably the nucleocapsid) of the Dane particle, a 43 nm virus-like particle antigenically related to hepatitis B antigen and thought to be the virus itself. In addition, a modified passive hemagglutination technique was developed. This technique provided a sensitive and specific means for identifying antibody to the various subtypes of hepatitis B antigen. It appears to have considerable value for epidemiologic study of the relative importance of the subtypes of HB Ag.

These and other serologic techniques were used for seroepidemiologic studies of HB Ag in an attempt to understand better the public health impact of hepatitis B infection. Longitudinal and cross-sectional studies of a number of populations in the United States and abroad revealed that hepatitis B infection was more wide-spread than was previously realized. The frequency of HB Ag antibody in several populations was found to be directly related to the age of individuals studied and inversely related to their socioeconomic status. There was little evidence for covert spread within healthy families

in the United States, but it appeared that both overt and covert infections were acquired from patients with acute hepatitis B infection in one group of families living in Panama. Medical personnel and others with exposure to hepatitis patients or their serum, tissues or excreta were also frequently found to have serologic evidence of covert infection with hepatitis B virus.

In addition to epidemiologic studies of human populations, an extensive serologic survey of non-human primates was carried out in an attempt to identify species of animals which were susceptible to hepatitis B infection. Primates exhibiting serologic evidence of prior infection were then studied intensively in an attempt to transmit hepatitis B infection under experimental conditions.

To date, infection has been transmitted successfully to chimpanzees, rhesus monkeys and a woolly monkey. Chimpanzees appeared to be approximately as sensitive as man to hepatitis B infection. Chimpanzees exhibited a spectrum of responses which ranged from inapparent infection to frank hepatitis; this pattern was analogous to that seen in man. It is likely that these animals will provide a means of studying the host response to hepatitis B virus infection. They appear to be the animals of choice for testing the efficacy and safety of potential hepatitis B vaccines. In collaborative studies with Dr. L. Barker (BOB) and Dr. J. Maynard (CDC) the infectivity titer of suspensions of human HBV, representing each of the recognized subtypes, are being determined; these virus suspensions will be used for cross-challenge experiments in an attempt to determine the significance of subtype antigens in resistance to infection and clinical hepatitis. In addition, the infectivity titer of partially purified Dane particles (thought to be the virus of hepatitis B) will be determined. These preparations will then be used for intensive attempts to isolate HBV in tissue culture.

Rhesus monkeys are relatively resistant to infection with human HBV, but when rhesus monkey-adapted strains of HBV are employed, this species of non-human primate provides a useful means for the characterization of HBV. Using such an adapted strain of HBV, an infectious suspension of virus has been prepared, titered and the host response characterized. This virus suspension is currently being used to characterize a number of biophysical and biochemical properties of HBV and to develop a neutralization test for the serologic study of infection. Preliminary experiments have shown that HBV was inactivated by heating at 100°C for 10 minutes or 80°C or 60°C for 1 hour. The virus also was inactivated by exposure to chloroform. Infection was successfully initiated by intravenous or intramuscular inoculation of infectious material but not by oral administration of the same suspension. A strong positive correlation was found between the detection of HB Ag in rhesus monkey serum and the infectivity of that serum for other rhesus monkeys.

Hepatitis B virus was successfully transmitted to one woolly monkey. The response of this animal was characterized by a seven month incubation period followed by HB Ag antigenemia of 3 months duration. An intense anti-HB Ag immune response was observed following disappearance of HB Ag from the serum. The serum transaminase values were transiently elevated during the period of antigenemia. Additional studies are in progress to determine the utility of woolly monkeys for hepatitis B research.

During the past year we attempted to identify virus-like particles in the stool of hepatitis A patients using the sensitive immune electron microscopy technique which had been used successfully to visualize the virus of acute, non-bacterial gastroenteritis. Unique virus-like particles were detected in the stool of a patient involved in an epidemic of viral hepatitis in the South Pacific. Antibody to this virus-like particle was detected in convalescent serum from patients involved in two separate viral hepatitis outbreaks and from individuals infected experimentally with hepatitis A virus, but an etiologic association of the particle with hepatitis A has not yet been established with certainty. The virus-like particle is currently being studied by a variety of biophysical and immunological methods (Purcell, Feinstone, Gold, and Moritsugu).

Acute Respiratory Tract Disease. Historically this is the oldest program in LID and it represents the area in which we have the best understanding of etiologic agents, natural history of infection and immunologic determinants of resistance. There are at least 120 different viruses, belonging to 6 different virus groups, and one mycoplasma which cause acute respiratory tract disease. Most of these agents were first recognized or characterized by workers in LID. Unfortunately, our advanced degree of understanding of acute respiratory tract disease has not been matched by our ability to prevent this type of illness. For this reason, recent efforts have been directed toward the development of effective immunoprophylactic measures for prevention of disease caused by the most serious and important respiratory tract pathogens-influenza virus, respiratory syncytial virus, parainfluenza viruses, certain of the adenoviruses and Mycoplasma pneumoniae.

Temperature Sensitive (ts) Mutants of Influenza Virus for Prevention of Influenzal Disease. Missence ts mutants of influenza A virus were prepared with the expectation that their temperature sensitive defects would restrict viral growth in vivo at the temperature of the lower respiratory tract (37°C), whereas replication should not be seriously impaired in the cooler environment of the upper respiratory passages (32-34°C). Ts mutants of a 1965 influenza A (H2N2) virus were produced by growing the virus in the presence of 5-fluorouracil. The ts lesions were then transferred to a Hong Kong influenza A (H3N2) virus by genetic recombination. Four such genetically distinct influenza A (H3N2) ts recombinants were evaluated for in vitro properties in tissue culture and for in vivo properties in the hamster and in man. The 4 ts recombinants exhibited a spectrum of temperature sensitivity; one recombinant had a shutoff temperature for plaque formation of 37°C, one shutoff at 38°C, while the remaining 2 recombinants had a shutoff temperature of 39°C. The 37°C shutoff recombinant did not infect seronegative volunteers. The 39°C shutoff recombinants infected each of the seronegative volunteers tested and appeared to be attenuated; however, attenuation was not complete since each of the volunteers developed respiratory tract symptoms, in some instances associated with low grade fever. The ts recombinant with intermediate temperature sensitivity infected almost all seronegative volunteers and appeared to be satisfactorily attenuated. Thus, there appeared to be a direct relationship between degree of temperature sensitivity in vitro and level of attenuation for man. In the initial volunteer studies it was shown that the 38°C recombinant, ts-1[E] induced complete protection to challenge with a virulent wild type suspension of Hong Kong virus. Furthermore, it was found that infection with the ts-1[E] virus did not spread from infected vaccinees to close contacts.

The response of volunteers to the attenuated influenza A (H3N2) ts-1[E] recombinant virus grown in bovine kidney (BK) tissue culture was examined in greater depth during the past year. In addition, the ts-1[E] recombinant grown in avian leukosis virus free eggs was given to seronegative volunteers and their response was compared to that of men given the bovine kidney culture grown virus. The egg grown recombinant was found to be attenuated, antigenic and genetically stable and did not differ perceptably in its effect on seronegative volunteers from the BK grown virus. Thus, the important properties of the ts-1[E] recombinant appeared to be genetically determined and not influenced by the host in which the virus was grown.

In the initial evaluation of the ts-1[E] recombinant $10^6 TCID_{50}$ of bovine kidney culture grown virus did not cause influenzal illness or lower respiratory tract symptoms. Attenuation of the virus was further confirmed recently by the finding that $10^7 TCID_{50}$ of egg grown virus produced a similar, benign clinical response, although virus replication was extensive enough to induce an immunologic response which approached that stimulated by the wild type, virulent virus. In addition several volunteers infected with $10^6 TCID_{50}$ of ts-1[E] virus had a level of virus replication in the nasopharynx which was comparable to that seen in volunteers who were infected with wild type virus and who developed influenzal disease. The ts-1[E] infected volunteers, in contrast, developed only mild upper respiratory symptoms.

It is likely that the absence of lower respiratory tract symptoms in the ts-1[E] vaccinees reflects a restriction in virus replication in the lungs. Previously we had shown that the ts-1[E] virus grew poorly in the hamster's lungs where the temperature is 37°C. Additional evidence that the lower respiratory tract is not involved during ts-1[E] infection was provided by the findings from a collaborative study performed with Dr. Gordon Douglas and colleagues. Seven young adults who had low or undetectable serum HI antibody for the Hong Kong virus were investigated. Each of the volunteers was infected following administration of $10^6 TCID_{50}$ of ts-1[E] virus; however, none of these individuals developed any alteration in pulmonary function over a 60 day period.

The ts-1[E] vaccinees, as a group, shed approximately 3000-fold less virus from the nasopharynx than volunteers infected with wild type virus, indicating that the recombinant was attenuated for the upper respiratory tract as well as the lower tract. In addition to attenuation and immunogenicity two other features make this virus a suitable candidate for further evaluation as a live virus vaccine strain: genetic stability and lack of communicability. To date, virus isolates from 44 volunteers administered the ts-1[E] virus have been examined for their temperature sensitivity, and each was shown to retain the ts phenotype, thus further documenting the genetic stability of this virus in man. The inability of the ts-1[E] virus to spread is probably due, in part, to its failure to induce cough or extensive sneezing and also its high human infectious $dose_{50}$ ($HID_{50}$) which appears to be approximately $10^5 TCID_{50}$. The high $HID_{50}$ of the ts-1[E] virus may be of particular importance since the quantity of virus produced in the nasopharynx is relatively small.

In a collaborative study performed with Dr. Parrott the ts-1[E] virus was given to 10 children, 3-1/2 to 11 years of age, who had low or undetectable serum HI antibody. Each of the children became infected but none developed a significant clinical response. A total of 73 adults with low or undetectable serum HI antibody have been given the ts-1[E] virus and their response to infection has been uneventful. In addition, 56 individuals, most of whom were elderly and most of whom had serum HI antibody, were studied for their response to the ts-1[E] recombinant.

The observations made during the past year further characterize the response of volunteers to the potential vaccine virus, ts-1[E]. The major picture that emerges from our experience is that the pattern of infection caused by the ts-1[E] virus is comparable to that found after infection with wild type virus but is of a lesser magnitude. This is an important consideration in the development and use of a live influenza virus vaccine because one would be justly reluctant to employ a mutagenized virus which manifested a pattern of infection which varied significantly from that of the wild type virus. However, this is not the case with the ts-1[E] virus.

The occurrence in 1972 of antigenic variation in the coat of the Hong Kong 1968 strain of influenza A provided an opportunity to study the feasibility of transferring both temperature sensitivity and attenuation of the influenza A/Hong Kong/68 (H3N2) ts-1[E] virus to a new strain within the H3N2 subtype. It is clear that the new virus is sufficiently different from the 1968 virus that optimal protection requires a vaccine which contains antigens that closely match those of the new virus. For this reason, we have isolated ts viruses with the hemagglutinin of the A/England/42/72 virus from a cross between our influenza A/Hong Kong/68-ts-1[E] virus and wild type influenza A/England/42/72 virus. The progeny from this cross were mixed with an appropriate antiserum monospecific for the 1968 hemagglutinin at a dilution that suppressed growth of the 1968 virus but permitted plaque formation by the 1972 virus. Plaques which developed at the permissive temperature (33°C) were picked and tested for temperature sensitivity. Of 30 plaques picked, 9 (30%) were temperature sensitive. The high frequency of recombination, reminiscent of the transfer of the ts lesions from the 1965 (H2N2) mutants to the 1968 (H3N2) virus described previously, demonstrated that temperature sensitive mutants with the desired surface antigens could be produced rapidly and readily in a single step. In vitro studies showed that the H3N2-68-ts-1[E] and H3N2-72-ts-1[E] viruses exhibited the same degree of temperature sensitivity as regards plaque formation in rhesus monkey and calf kidney monolayer cultures. This result again demonstrates that the level of temperature sensitivity exhibited by the parent and recombinant ts viruses is determined by the ts lesion and not by other factors present in the recipient virus. Studies in mice showed that the recombinant 1972-ts-virus exhibited restricted replication in mouse pulmonary tissue, like the parent 1968-ts-1[E] virus.

Influenza B virus is a respiratory pathogen which infects all age groups and is associated with excess mortality in some years which is comparable to that caused by influenza A virus. We have therefore begun to develop a live virus vaccine using the methods employed with influenza A virus. We have mutagenized influenza B/Maryland/1959 virus by growing the virus in the presence of 5-fluorouracil. Approximately 400 plaques have been characterized for temperature

sensitivity and 10 appear to have genetically stable ts defects. These 10 ts viruses are currently being cloned. The temperature sensitivity of these viruses ranged from 37°C to 39°C. Large suspensions have been prepared for genetic analysis, animal studies, and recombination with current influenza B viruses (Murphy, Richman, Stewart and Chanock).

Mycoplasma pneumoniae. During experimental infection of the hamster with M. pneumoniae the site of localization of the organism appears to be superficial, involving only the epithelial layer of the respiratory passages. The superficial nature of M. pneumoniae infection in the lower respiratory tract suggests that local immune mechanisms (secretory IgA and/or cell mediated immunity) in the respiratory tract should be of greater importance than serum antibody in protection from disease. Recently, the development of a highly sensitive radioimmunoprecipitation (RIP) assay made it possible to detect the development of specific IgA antibody for M. pneumoniae in the nasal secretions of a majority of volunteers infected with this organism. Previous attempts to detect antibody in local secretions had failed but this failure may have resulted from an insensitivity of the serologic methods used. We used our new radioimmunoassay to measure the level of IgA M. pneumoniae antibody in the pre-infection nasal washings collected from 45 volunteers who were challenged with wild type M. pneumoniae by Dr. Robert Couch and colleagues. Men with low levels of RIP nasal antibody (<1:3) developed M. pneumoniae disease with higher frequency following experimental challenge than did men with a higher titer (>1:3), irrespective of serum titer. In contrast a correlation of serum antibody with the resistance was not evident. These findings suggest that local IgA antibody as measured by the RIP test was related to resistance to M. pneumoniae disease and was more important than serum antibody.

Because of the finding that secretory antibody may play a critical role in immunity a preliminary examination of the effect of formaldehyde killed vaccine instilled intranasally was made. Hamsters were given 3 separate doses of 0.2 ml of vaccine (lot #0SUIB) intranasally or subcutaneously over a 2 week period. Wild type infection and broth control groups were also included. Two weeks following the last administration of vaccine the hamsters were challenged with wild type organisms and were sacrificed 10 days later. There was a significant decrease in pulmonary lesions in the intranasally vaccinated animals although little if any effect on growth of the organism in the lungs was seen. The effect of IN vaccine on prevention of pulmonary lesions was as great as that produced by prior infection with wild type organisms. Because of these promising findings, a preliminary human volunteer trial is planned.

Because of the promising results observed in studies performed in hamsters with the ts 640 mutant, this organism was evaluated in eleven volunteers who lacked serum MI antibody. In hamsters the ts 640 mutant produced an extensive infection without inducing pulmonary lesions. Further, the mutant was stable genetically and induced significant resistance to wild type challenge. The ts 640 mutant, which was of intermediate temperature sensitivity, infected each of the volunteers and most of the infections were silent. Furthermore, each of the isolates recovered from the volunteers was temperature sensitive, indicating that the ts 640 mutant was stable genetically in man. However, 2 of the volunteers developed bronchitis which indicated that the

mutant possessed sufficient residual virulence to disqualify it as a candidate vaccine strain. Nonetheless, the results of the volunteer study were sufficiently encouraging to prompt us to continue our clinical evaluation of ts mutants of M. pneumoniae which are more temperature sensitive than ts 640.

A search for glycolipid antigenic determinants shared by M. pneumoniae, vegetables, and certain bacteria was prompted by the observation that an unexpectedly large proportion of infants and young children possessed antibody to Mycoplasma pneumoniae in their serum. Since early childhood represents a time of life when the organism is not commonly associated with lower respiratory tract disease, the origin of antibody possessed by infants and young children was not immediately apparent. Such antibody could be the result of clinically inapparent infection which occurred with high frequency. There is some evidence from epidemiologic studies performed at Children's Hospital of D.C. that such a silent infection may occur with appreciable frequency during the pre-school period. On the other hand, cross-reactive or shared antigenic determinants might be responsible for some of the childhood serum antibody which reacts with M. pneumoniae. During the past year we have focused on the latter possibility and have investigated the capacity of antigenic determinants from other sources to stimulate antibody reactive with M. pneumoniae. In the initial tests chloroform-methanol (7:3) eluates containing mixed lipids from 53 vegetables were evaluated for their capacity to fix complement with antibodies to M. pneumoniae or its glycolipids. Forty-three of the vegetables were found to have antigenic activity with the M. pneumoniae immune sera used. Each positive vegetable reacted with the rabbit anti-M. pneumoniae glycolipid serum. Eighteen gram positive bacterial or L forms were also studied and 3 fixed complement with the M. pneumoniae antisera. The positive reactors included Staphlococcus aureus, Staphlococcus aureus L form and Streptococcus hemolyticus group A, type I L form

Two of the vegetables which exhibited a high level of antigen activity were examined in greater detail. Purified glycolipids from carrots and parsnips as well as artificial membrane reaggregates containing these glycolipids exhibited a high degree of complement fixing activity when tested with antibodies directed against M. pneumoniae glycolipids. In addition, carrot and parsnip glycolipids incorporated in A. laidlawii membrane reaggregates were found to be efficient in blocking mycoplasmacidal antibody contained in human convalescent serum and in rabbit antiserum prepared against the glycolipids of M. pneumoniae.

The preceding experiments indicated that vegetables contained lipid or glycolipid antigens which reacted with antibodies stimulated by M. pneumoniae. It was of interest to determine if the reverse were true, namely could vegetable glycolipids stimulate M. pneumoniae antibodies? Carrot and parsnip glycolipids, either in a suspension of lecithin or incorporated into an artificial A. laidlawii membrane reaggregate, stimulated antibodies to M. pneumoniae which could be detected by CF, metabolism-inhibition and mycoplasmacidal assay. As expected glycolipid membrane reaggregates were more immunogenic than the glycolipid suspension alone. Although the chemical configuration of the carrot and parsnip glycolipids awaits further analysis, it is reasonable to predict at this time that the active glycolipids are probably similar in structure to their M. pneumoniae counterparts based upon immunological

reactivity, blocking of mycoplasmacidal antibody activity and stimulation of antibodies which react with M. pneumoniae glycolipids (Greenberg, and Chanock).

Respiratory Syncytial Virus. Research on respiratory syncytial virus (RSV) proceeded along several lines: (1) further characterization of existing ts mutants of the virus; (2) production of new mutants; (3) studies of the mechanism of pathogenesis of the virus and of the mechanisms of host resistance; (4) examination of basic biologic properties of the virus and of its infectious process in cells.

A stable clonal population of the ts-2 mutant was reisolated after replicate tests revealed an irregularity in plaque morphology at permissive temperature. The reisolated clonal population of ts-2 virus failed to produce plaques at restrictive temperature and reproducibly exhibited the altered plaque morphology characteristic of the mutant. Using this clone, the plaque complementation studies described in 1971-1972 were completed, and the mutants were grouped into three complementation groups. One group was represented by the 5-FU induced mutants, ts-1, ts-3, ts-4, ts-5 and ts-6. A second group was represented by the ethyl-methanesulfonate (EMS) induced mutant, ts-7. These two groups had already been elucidated by earlier growth complementation experiments and were further confirmed by plaque complementation. Ts-2, which exhibited considerable growth at restrictive temperature (leakiness) as well as altered plaque morphology, was demonstrated to belong to a third complementation group. Cells doubly infected with ts-2 and with a mutant belonging to either of the other complementation groups, produced plaques when incubated at restrictive temperature. In contrast, cells singly infected with any of the mutants or cells doubly infected with mutants belonging to the same complementation group, produced no plaques when incubated at the same restrictive temperature. The leakiness of ts-2 had previously prevented its characterization in growth complementation experiments.

Further refinements were undertaken in the step-up experiments and, in addition, step-down experiments were performed. The results of these experiments were reproducible and indicated that ts-1, ts-3, ts-4, ts-5 and ts-6 mutants, which belong to a single complementation group, were all defective in a function operative late in the growth cycle. In contrast, ts-7, which formed a separate complementation group, was deficient in an earlier function. The step-up and step-down experiments supported the classification of mutants arrived at through complementation studies. Ts-2 showed too much leakage in the step-up and step-down experiments to allow for accurate determination of defective segment of its growth cycle (Hodes and Chanock).

New Techniques for Recovery of Respiratory Tract Viruses. Specimens obtained from patients in the NIH, LID Common Cold Study have been studied intensively for the presence of viruses by both conventional and new techniques. The specimens were first examined by routine cell culture methods, and only those which failed to yield viruses were studied further in experimental culture systems--such systems include new cell cultures and human embryonic tracheal (HET) organ cultures. The use of new culture systems has been especially fruitful in the recovery of virus strains belonging to the human

coronavirus group. Sixteen of the 32 described coronavirus strains were first recovered by this laboratory employing new culture systems. However, study of the coronaviruses has been hampered by the lack of convenient and suitable methods for their propagation. Ten of the 32 coronavirus strains were originally isolated in HET organ cultures only, while the remaining 22, all of which were similar to a prototype strain designated 229E, were isolated in monolayer tissue cultures. Since the coronaviruses appear to be important etiologic agents of adult upper respiratory tract illnesses, continued intensive efforts have been made to develop new ways to detect these agents. Nasopharyngeal washings were carried through 3 passages in HET OC and the harvests of the third passage examined for the presence of virus particles utilizing in addition to conventional electron microscopy, the technique of immune electron microscopy, in which the patient's convalescent serum is the source of specific antibody. Immune electron microscopy was employed so that virus particles would appear in the form of "aggregates" rather than individual units thereby facilitating the visualization of virus present in low titer. With this and conventional techniques as well, a new coronavirus strain was detected which grew only in organ culture. The relationship of this virus to 229E and OC43 prototypes will be investigated by immune electron microscopy. It is also noteworthy that 9 additional coronavirus strains similar to 229E have been detected in monolayer culture systems in this laboratory.

In addition, 5 fastidious rhinoviruses have been recovered by the combined organ culture--monolayer culture technique. Washings were first inoculated into organ cultures of human fetal trachea and passaged 3 times in this system. Harvest fluids from the 3rd organ culture passage were then inoculated into monolayer cultures. Five strains of rhinovirus were detected in human fibroblast cultures by this method although these cultures did not exhibit cytopathic effect when inoculated with the original washings. Of interest was the finding that 4 of the 5 rhinovirus isolates belonged to serotypes which had been recovered in monolayer cultures previously (Kapikian and Kalica).

Serial No. NIAID 66A
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Epidemiologic Studies of Infections and Illness, and Their Control in Infants and Children

Previous Serial Number: 66A-B

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: Mr. W. Lee Cline, Serology Unit, LID, NIAID

Contract Support: None

| Man Years: | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 4/12 | | | 4/12 |
| Professional: | <1/12 | | | <1/12 |
| Other: | 4/12 | | | 4/12 |

Project Description:

This was a long term, intensive study of the illness and microbial experience of infants and children at Junior Village, a District of Columbia Welfare Institution. This study began in July of 1955 and the field portion of the study was discontinued on May 21, 1969.

Basic Objectives:

The general objective was to maintain under observation a population group suitable for epidemiologic study of occurrence of infections and disease and, in addition, to study host-parasite-disease relationships as they occur naturally and as they could be altered by vaccines and/or chemo-prophylactic agents. A concerted effort was made to (a) find new microbiologic agents which cause disease; (b) find methods of identification of these agents; (c) define modes of spread of infectious agents; and (d) find methods for prevention and treatment of acute illnesses, particularly respiratory illnesses.

Methods Employed and Patient Material:

The field portion of this study was discontinued on May 21, 1969. The epidemiologic setting was described in detail in previous annual reports.

Currently, most of the original throat swab specimens from the study are stored at -70°C for the study of coronaviruses when suitable methods for their propagation become available; in addition, anal swab specimens are stored at -20°C for the study of gastroenteritis agents when suitable methods for their propagation are found.

Major Findings:

Epidemiology of Rhinovirus Infection and Characterization of Rhinoviruses - Most respiratory viruses have been studied extensively at Junior Village. However, one major group which has not been studied extensively has been the rhinoviruses since antisera were not available for many serotypes and as a result typing of strains on a limited basis was generally unsuccessful. However, with the completion of the second phase of the rhinovirus collaborative program and the availability of antisera to all 89 serotypes and the one subtype, efforts will be made to study the epidemiology of the numerous rhinovirus serotypes at Junior Village. Thus far, acid lability tests have been carried out on 344 viral isolates from 222 residents and 122 viruses have been found to be acid labile. These studies will be continued in an attempt to determine the epidemiologic pattern of rhinovirus infection in this population.

An Outbreak of Infectious Lymphocytosis - This outbreak occurred in a four month period when at least 22 cases of acute infectious lymphocytosis were discovered. The leukocyte counts varied from 20,000 to 94,000 per mm. with a relative lymphocytosis on the average of approximately 80%. Routine viral, bacterial, fungal, and parasitic studies were performed on specimens obtained during the outbreak and no definitive etiologic agent was found. Renewed attempts will be made to isolate or detect an etiologic agent by using newer techniques such as organ cultures, experimental cell cultures, and immune electron microscopy. The occurrence of EB virus infection in 10 children with infectious lymphocytosis was studied using the immunofluorescent technique. These studies which were reported in a previous annual report revealed that none of 6 children without pre-existing antibody, and none of 4 with such antibody developed four-fold or greater EB virus antibody rises. In addition, paired sera from 9 of the 10 children were tested by the CF technique for evidence of infection with cytomegalovirus (CMV) and herpes simplex virus (HSV); no rises to CMV and one to HSV were detected.

Seroepidemiological Studies of Coronavirus Infections - In a previous annual report we reported the incidence of OC43 and 229E virus infections as determined by serologic (CF) tests of paired sera obtained from children admitted to Junior Village between October 15 and December 15 during the years 1961 through 1968. This period had been selected in an attempt to bracket the "winter" seasons with a serum collected on admission to the cottage and a serum taken several months later since it had been shown that adult coronavirus infections were prevalent during both a "winter" and "winter-early spring" period. Since it was found that 23 (16%) of 148 children developed significant CF antibody rises to OC43 virus (in contrast, however, only 1 of 149 developed such a 229E virus antibody rise), these seroepidemiologic

studies have been extended to include sera obtained during all periods of the year. In addition, in these expanded studies, the sera have been tested not only by the CF technique for 229E and OC43 virus antibody, but also by the HI technique for OC43 virus antibody. A preliminary comparison of the sensitivity of the HI and CF techniques for detection of OC43 and OC43 virus-related infection was made: 12 antibody rises were detected by the CF and/or HI techniques--5 of these were detected by the HI technique only and 3 by the CF technique only indicating that both are essential for maximum detection of infection. In addition, study of serially collected sera tested by both techniques indicated that OC43 virus HI antibody persists for longer periods than CF antibody. The relative sensitivity and specificity of these serologic techniques for detection of OC43 virus infection must await the development of sensitive OC43 virus isolation techniques. These seroepidemiologic studies will be continued and further analysis made in an attempt to determine the importance of coronaviruses in this population. Selected throat swab specimens will also be tested in HEI MA177 cell cultures in an attempt to recover coronaviruses from those periods shown by serologic tests to have had a high incidence of coronavirus infections.

Significance to Biomedical Research and the Program of the Institute:

This project represents a systematic approach to determining the various etiologies of acute febrile illnesses. Since carefully gathered epidemiological data and clinical specimens are available, this project also offers an unusual opportunity for the study of the epidemiologic patterns of infections associated with newly discovered agents.

Proposed Course of Project:

The field portion of this study was discontinued on May 21, 1969, when it became apparent that vaccine studies could not be carried out in this population. Studies relating to this project will be continued as long as profitable.

Honors and Awards: None

Publications: None

Serial No. NIAID 66H
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Etiology of Acute Infectious Nonbacterial Gastroenteritis

Previous Serial Number: Same

Principal Investigators: Dr. Richard G. Wyatt
Dr. Albert Z. Kapikian
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Other Investigators: Dr. Robert M. Chanock

Cooperating Units: Dr. Saul G. Agus and Dr. Z. Myron Falchuk (NIAMDD, D&HD)
Dr. Lewellys F. Barker (BOB)
Dr. Raphael Dolin (NIAID, LCI)
Dr. Herbert L. DuPont and Dr. Richard B. Hornick (Univ. of Maryland)
Dr. John L. Gerin (MAN Laboratory)

Contract Support

| Contract Project Number | Project Officer (NIAID) | Contractor | Contractor's Project Director | Annual Funding Level |
|---|---|---|---|---|
| PH43-68-1309 Part B | Dr. Chanock | Flow Labs | Dr. Gilden | $ 202,577 |
| NIH-NIAID 73-2505 | Dr. Chanock | University Hospital Boston, Mass. | Dr. N.R. Blacklow | $ 20,000 |

| Man Years: | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 81/12 | | | 81/12 |
| Professional: | 31/12 | | | 31/12 |
| Other: | 50/12 | | | 50/12 |

Project Description

Objectives: (1) To search for viruses which may play an etiological role in the syndrome of acute infectious nonbacterial gastroenteritis; (2) to cultivate (in vitro) the viral agent(s) of acute infectious nonbacterial gastroenteritis; (3) to characterize such agent(s) by studying their biophysical, immunological, and epidemiological properties; (4) to reproduce

the syndrome experimentally in volunteers for the purpose of a) studying the pathophysiological and immunological responses of the host, and b) assaying the infectivity of such viral agents.

Methods Employed

(1) Source of Clinical Materials: Clinical materials were collected from a variety of recent naturally occurring outbreaks of acute infectious nonbacterial gastroenteritis: a) Willow Lake, Oregon, July 1972 (Courtesy of Dr. Claude Friedman); b) Walla Walla, Washington, Sept. 1972 (Courtesy of Dr. James Koopman); c) Seattle, Washington, October, 1972 (Courtesy of Dr. Michael Daly); and d) Shippensburg, Pennsylvania, November, 1972 (Courtesy of Dr. Michael Mercen). Additional previously studied infectious material derived from an outbreak of gastroenteritis in England in 1963 was made available through the courtesy of Dr. David Tyrrell (Clarke, S.K.R. et al, Brit. Med. J. 3: 86-89, 1972). Clinical materials from earlier outbreaks of gastroenteritis were available for continued study. These included specimens from a) USS Shenandoah; b) Norwalk, Ohio; c) Denver, Colorado; d) Tampa, Florida; e) New Britain, Connecticut; f) Morgantown, West Virginia; g) Montgomery County, Maryland; h) Bethesda, Maryland; i) Henryton, Maryland; and j) Honolulu, Hawaii.

(2) Preparation of Fecal Samples: Stool suspensions were made in veal infusion broth with 0.5% bovine serum albumin, clarified by low-speed centrifugation, and filtered through 1200 nm and 450 nm Millipore membranes to exclude bacteria. These filtrates were then utilized for a) inoculation of tissue culture and intestinal organ culture, b) examination by electron microscopy, c) particle purification and characterization and d) administration to volunteers following rigorous safety tests in tissue culture, laboratory animals, and other systems to exclude adventitious agents.

(3) Culture Systems: a) Fecal specimens from individuals with naturally occurring or experimentally induced disease, as well as organ culture harvests, were inoculated into a variety of primary and continuous monolayer tissue cultures at times when the cells were rapidly dividing; propagation of virus in such systems was attempted at 34°C and 37°C., at pH 6.7 and pH 7.1, and with incubation in both stationary racks and on roller drums. Type and quantity of serum added to growth and maintenance media for these studies were varied. Tissue culture harvests were serially passaged, and inoculated cultures were examined for cytopathic effects and hemadsorption.

b) Two different lines of cell suspension cultures were inoculated with fecal samples as described above. Cell growth patterns were observed and cells were examined periodically for fluorescent stainable antigen.

c) Human fetal intestinal organ cultures were again utilized in attempts to cultivate viral agents from gastrointestinal disease. Fecal samples derived from naturally occurring outbreaks and experimentally induced disease were inoculated into intestinal organ cultures, and extracellular fluids were passaged serially. Tissue explants were observed for presence of

fluorescent stainable antigen, and extracellular fluids were examined by immune electron microscopy for virus particles. Organ culture fluids derived from serial passage of fecal filtrates in this system were also administered to volunteers as described below; the same rigorous safety tests were applied to these fluids as were used for fecal filtrates.

(4) Immune Electron Microscopy: Stool filtrates from infected volunteers who developed gastroenteritis were examined by immune electron microscopy using the convalescent serum from ill volunteers as the source of specific antibody. This approach was taken in the hope that antibody would aggregate virus particles thereby facilitating visualization of a small virus, possibly present in low titer.

(5) Biophysical Studies: To determine the buoyant density of the 27 nm 8FIIa particle, an aliquot of a Norwalk-derived stool filtrate (8FIIa) was concentrated (10X), unless otherwise noted, using the Amicon Ultrafiltration system. This concentrate was used as the starting sample in either a discontinuous density gradient of cesium chloride or potassium tartrate and banded isopycnically in an SW41 rotor on a Beckman L3-50 centrifuge. The gradient was fractionated into 16 equal fractions (.75 ml). Each fraction was diluted (1:3.33) in PBS and examined by immune electron microscopy using an appropriate convalescent serum. The number of 27 nm particles seen in each fraction was expressed as a percent of the total number of particles seen in the entire gradient.

To determine the buoyant density of the infectious particle, fractions from a potassium tartrate gradient were pooled to give density ranges of (1) 1.50-1.41 g/cm$^3$, (2) 1.37 - 1.28 g/cm$^3$, and (3) 1.28 - 1.00 g/cm$^3$. Each of the three samples was rediluted with PBS to the original unconcentrated 8FIIa volume used in the starting sample. Further safety tests were performed to assure absence of adventitious agents. Aliquots of each of the potassium tartrate gradient pools were given to volunteers.

(6) Nonhuman Primate Studies: Earlier attempts to reproduce the syndrome of acute infectious nonbacterial gastroenteritis in a variety of laboratory animals including rhesus monkeys failed, although the same fecal filtrate derived from the Norwalk, Ohio outbreak produced illness in volunteers. Chimpanzees were then administered this same inoculum by stomach tube and observed for signs of illness. Stool and serum samples for further study were collected from these animals at regular intervals.

(7) Volunteer Studies: Between 1970 and 1973 volunteer studies have been conducted to ascertain the presence of infectious transmissible material in the fecal filtrates derived from various outbreaks of acute infectious nonbacterial gastroenteritis. Attempts have been made to define biological and physical properties of one such agent, i.e. Norwalk, and the patho-physiological and immunological responses to selected agents causing this syndrome have been examined. To date, 306 volunteers have been studied at the Maryland House of Correction, Jessup, Maryland, at the General Clinical Research Center, University Hospital, Boston, Massachusetts, and at the NIH

Clinical Center. In all cases experimental disease has been entirely self-limited, uncomplicated, and has resolved spontaneously.

Major Findings:

I. Further Studies with Norwalk Agent of Acute Infectious Nonbacterial Gastroenteritis -

(A) Summary of previous findings: In 1970-71 acute infectious nonbacterial gastroenteritis was transmitted to volunteers by the administration of a stool filtrate from the Norwalk outbreak (Dolin, et al., J. Infect. Dis. 123: 307-312, 1971). The original material has been transmitted through three serial passages in volunteers; characterization of the Norwalk agent in volunteers indicated (a) immunity to rechallenge with the same inoculum 6-14 weeks was seen after prior experimental illness, (b) the etiologic agent was a small (probably less than 36 nm), ether and acid resistant, and relatively heat stable virus, (c) the host range was restricted to man, (Dolin, et al., Proc. Soc. Exp. Biol. Med. 140: 578-583, 1972), and (d) reversible histopathologic changes developed involving the jejunal mucosa of ill volunteers (Agus et al., Ann. Intern. Med. [in press], 1973).

(B) Immune electron microscopic studies of a Norwalk outbreak-derived filtrate(8FIIa): The 2% second human passage stool filtrate (8FIIa) used in these studies was derived from stool specimens from a volunteer who developed gastroenteritis after oral administration of a stool filtrate derived from one of the two volunteers who became ill after receiving the original inoculum from the Norwalk outbreak. We examined this filtrate for the presence of virus particles by immune electron microscopy utilizing uninactivated convalescent serum from experimentally infected volunteers as the source of specific antibody. The serum-stool filtrate and serum-PBS control mixtures were routinely incubated at room temperature for 1 hour; PBS was then added, if necessary, to make a final pre-centrifugation volume of 1.0 ml. The mixtures were then centrifuged at 17,000 rpm for 90 minutes; the supernatant fluid was discarded and the pellet was prepared for examination by negative staining (3% phosphotungstic acid) in the electron microscope.

Utilizing this technique, we were able to visualize in the stool filtrate aggregates comprised of virus-like particles which were heavily coated with antibody; these particles were not randomly distributed but were present as groups which stood out clearly from the surrounding matter, and resembled the picorna- or parvoviruses morphologically. In stool filtrate-PBS control preparations occasional particles or groups of particles without apparent antibody were seen; they measured approximately 27 nm in their shortest diameter and 32 nm in their longest and also resembled the picorna- or parvoviruses. The significance of these particles would have been difficult to evaluate without the previous experience acquired from examining similar but heavily coated particles which had been aggregated by antibody.

These studies were then extended to include both pre-challenge and convalescent sera in an attempt to detect serological evidence of infection by immune electron microscopy. In these studies, the grids were read without prior knowledge of the specimen being examined, and the quantity of the antibody on the particles in an aggregate was rated from 1 to 4+; a 1+ difference was considered to be a significant change in the amount of antibody present. Paired sera from 5 volunteers challenged with a Norwalk-derived filtrate were examined; 4 of the 5 who developed illness following challenge developed serological evidence of infection whereas the one volunteer who did not become ill did not develop such evidence of infection.

Although all four volunteers who developed illness after challenge with the Norwalk-derived filtrate developed serological evidence of infection, it was possible that the observed particles might represent a virus which was not the etiological agent of the Norwalk outbreak as it was conceivable that an adventitious virus could either have been present in the stool of the donor patient from the original Norwalk outbreak or could have been acquired during passage through volunteers. We examined this possibility by testing paired sera from 6 individuals from the original Norwalk outbreak; 4 were primary cases, one a secondary case and one a contact who did not become ill. Three of the 5 persons with naturally acquired Norwalk gastroenteritis developed an increase in antibody to the 27 nm particle during convalescence from disease,whereas the two who did not show such an increase demonstrated a high level of antibody in both their acute and convalescent sera. The acute phase sera were not collected until several days after the onset of disease and this may explain our failure to detect a response in two of the patients. The contact who did not become ill did not exhibit an increase in antibody. Among the seroresponders was the donor of the original rectal swab specimen which had induced illness in 2 of the 3 volunteers in the initial volunteer study. We examined by immune electron microscopy the pre-challenge and convalescent sera of these 2 volunteers and found that one developed an increase in antibody whereas the other did not. The development of an increase in antibody by 3 of the 5 naturally ill individuals suggested that the 27 nm particle was not acquired during passage in volunteers. Furthermore, these serum responses indicated that the 27 nm virus-like particles had infected individuals in the Norwalk outbreak.

Finally, we investigated the possibility that gastrointestinal disease might stimulate the observed antibody responses by a nonspecific mechanism. We examined by immune electron microscopy the sera of volunteers who were first administered either a stool filtrate containing an infectious agent from a secondary case of gastroenteritis which had occurred in Honolulu, ("H"), Hawaii or in Montgomery County, ("MC"), Maryland. Those volunteers who became ill after primary challenge were later rechallenged with the same agent, and homologous immunity was demonstrated. Subsequently challenge with a filtrate derived from the Norwalk outbreak produced disease and indicated that the filtrates derived from the Hawaii and Maryland cases differed from that of Norwalk. Sequential sera from a volunteer who underwent the "H", "H", Norwalk, and a volunteer who underwent the "MC","MC", Norwalk-derived filtrate sequence of challenges just described were studied by immune electron

microscopy with the 8FIIa filtrate as antigen. The volunteer who was administered the "H"-derived filtrate failed to develop an increase in antibody following gastroenteritis induced by such primary challenge but did develop a significant response after illness induced by the Norwalk-derived filtrate suggesting that the immune electron microscopy response was specific. The other volunteer developed an increase in antibody after gastroenteritis induced by primary challenge with the "MC"-derived filtrate and a further increase after illness induced by the Norwalk-derived filtrate suggesting that the infectious agents in the "MC" and Norwalk-derived filtrates may have been antigenically related.

It was noteworthy that each of the 13 individuals demonstrated the presence of antibody in pre, acute phase or early sera, suggesting that infection with the agent derived from the Norwalk outbreak or a related agent (or agents) was quite common. Possibly the agents of nonbacterial gastroenteritis may resemble certain respiratory viruses in their capacity to reinfect with facility.

These data suggested that the 27 nm particle was the etiological agent of Norwalk gastroenteritis. Although it is conceivable that the 27 nm particle induced infection which was not related to the disease, it is quite unlikely. In any case, additional laboratory and epidemiological studies are needed to confirm the postulated etiological relationship.

(C) Buoyant density in cesium chloride of the 27 nm particle: A 3 ml concentrate (10X) of 8FIIa was banded isopycnically in a discontinuous density gradient of CsCl and the fractions were treated as described in the Methods section. Visual examination of the tube following centrifugation revealed the presence of a discrete light-scattering band at a position identical to that of a 1.40 g/cm$^3$ density bead added to the companion balance tube. Examination of each of the gradient fractions by immune electron microscopy revealed at least one aggregate in 4 of the 16 fractions: one in fraction 3; 8 in fraction 4; 8 in fraction 5; and one in fraction 13. Aggregates observed in fraction 4 on the average consisted of a greater number of particles than those in the other fractions. As a quantitative estimate of the distribution of the 27 nm particles in the density gradient, the number of particles comprising each of the aggregates in each of the four positive fractions was determined from electron micrographs and the results for each fraction were expressed as a percentage of the total number of particles recovered (Micrographs of 17 of the 18 aggregates were available; the unphotographed aggregate was in fraction 4 and for this tabulation was assumed to contain 3 particles--the minimum number necessary to be considered as an aggregate.) On this basis, of the 463 particles tabulated, 12 (2.6%) were in fraction 3, 301 (65.0%) in fraction 4, 125 (27.4%) in fraction 5, and 23 (5.0%) in fraction 13. This method of quantitation yields a symmetrical distribution of the 8FIIa particles about a peak concentration in fraction 4 with a buoyant density of 1.403 g/cm$^3$ in CsCl. The small percentage of particles recovered in the starting sample zone (fraction 13) might be explained by physical trapping of some particles in lipid-containing material in the stool filtrate. In two additional isopycnic banding experiments in

CsCl a discrete light-scattering band was again observed in each tube following centrifugation at a position identical to that of a 1.40 g/cm$^3$ density bead added to each companion balance tube; the peak fraction of 8FIIa particles as determined by immune electron microscopy was found at a density of 1.413 g/cm$^3$ utilizing an unconcentrated 8FIIa filtrate, thereby confirming the initial experiment.

These results indicate that the 27 nm 8FIIa particle possesses a buoyant density of 1.38 - 1.41 g/cm$^3$. On this basis it would appear that the 8FIIa agent is not an enterovirus, the latter having a lower buoyant density in CsCl. Although the 8FIIa agent has a density in CsCl similar to that of a rhinovirus, it can be excluded from this subgroup of the picornaviruses also, since rhinoviruses are acid labile whereas an infectious stool filtrate derived from the Norwalk outbreak has been shown previously to be acid stable. Although the nucleic acid content of the 8FIIa agent has not yet been determined, it appears that this agent is "parvovirus-like" since it not only has a similar buoyant density in CsCl but also shares other properties with the parvoviruses such as morphology, and acid, ether, and relative heat stability. It is noteworthy that if our previously described study, which presented evidence for an etiologic relationship between the "8FIIa" agent and Norwalk gastroenteritis is confirmed, this presumed "parvovirus-like" agent will represent the first such agent to have been associated etiologically with human disease.

(D) Buoyant density of the infectious particle in the 8FIIa filtrate: Oral administration of sample 1 (density range of 1.50 - 1.41 g/cm$^3$ in potassium tartrate) produced no clinical illness in 4 volunteers. Three of eight volunteers receiving sample 2 (1.37 - 1.28 g/cm$^3$) developed mild clinical illness and 2 of 4 volunteers receiving sample 3 (1.23 - 1.0 g/cm$^3$) became ill. The clinical illness was more severe in the volunteers receiving sample 3 as compared to those receiving sample 2.

Immune electron microscopy revealed fourteen percent of the 27 nm 8FIIa particles in the density range corresponding to sample 2 given to volunteers and eighty-six percent was found in the region corresponding to sample 3. No particles were found in the region corresponding to sample 1. Sixty-six percent of the 27 nm particles was found by immune electron microscopy at a buoyant density of 1.04 g/cm$^3$ in potassium tartrate and a visible light scattering band was seen at that region. One explanation for this spurious banding in potassium tartrate is that of physical trapping of the particle by lipid containing material in the stool filtrate. Current studies are underway to elucidate this problem.

(E) Development of a serological assay: Hemagglutination of rat erythrocytes is seen with the 8FIIa pool but it occurs as well using control stool. Attempts at hemagglutination inhibition using paired sera have failed. Both complement fixation and radioimmunoassay using convalescent sera from volunteers are nonspecific.

In order to develop specific high titer antibody to the 27 nm gastroenteritis particle, the 1.40 g/cm$^3$ density region of doubly banded 8FIIa in cesium chloride was inoculated into guinea pigs. Using immune electron microscopy, antibody to the 27 nm particle has been detected in the hyperimmune serum whereas no antibody is found in pre-inoculation guinea pig serum.

Currently, this hyperimmune serum is being used in systems of hemagglutination-inhibition, complement fixation, and countercurrent electrophoresis in order to develop a specific serological assay for the 27 nm particle.

(F) Heterologous immunity studies in volunteers: Infectious stool filtrates derived from two other outbreaks of acute infectious nonbacterial gastroenteritis were used along with Norwalk-derived filtrates in cross challenge studies. A filtrate derived from a family outbreak in Montgomery County ("MC") produced disease in 2 of 4 volunteers; when a similar filtrate, made from diarrheal stool of one of these men, was studied, it produced disease in 3 of 14 volunteers. A filtrate from another family outbreak in Honolulu, ("H") Hawaii, produced disease in 3 of 4 volunteers; when a filtrate was made from a stool from one of these ill men, it produced illness in 5 of 12 volunteers. With both "H" and "MC" filtrates, immunity to homologous rechallenge was demonstrated 6-29 weeks after primary challenge. Nine men, each of whom developed illness on primary challenge with either Norwalk or "H" derived stool filtrates, were rechallenged with the heterologous filtrate 7-15 weeks later; five became ill with typical acute infectious nonbacterial gastroenteritis following the second challenge. Ten volunteers each of whom developed illness on primary challenge with either Norwalk or "MC"-derived stool filtrates were rechallenged with the heterologous filtrate 6-8 weeks later. Only one man became ill with the typical syndrome after the second challenge. This suggests therefore that the Norwalk agent is antigenically different from the "H" agent and may have some degree of relatedness to the "MC" agent.

(G) Nonhuman primate studies: In collaboration with Dr. Lewellys Barker, studies with chimpanzees were begun. Two animals were given orally stool filtrate (8FIIa) known to contain the 27 nm Norwalk particle described above and known to cause disease in 19 of 32 (59%) human volunteers unselected for pre-existing immunity. Neither animal developed clinical gastrointestinal illness, although one demonstrated anorexia and malaise at 36 hours after inoculation. Paired sera, pre-, and 4 weeks convalescent, were evaluated by the immune electron microscopic technique described above, and both showed a definite and significant serological response to the Norwalk particle. In further studies we plan to inoculate (a) additional chimpanzees in an attempt to produce illness with gastrointestinal signs, and (b) rhesus monkeys to attempt to detect a serologic response to the Norwalk agent.

(H) Further clinical studies with Norwalk agent: Histopathologic changes have been demonstrated previously in volunteers ill with Norwalk-induced disease. Light and electron microscopic alterations in jejunal mucosal cells included blunting of villi, disorganization of epithelial lining cells, cytoplasmic vacuolization, increased lysozomal bodies, and widened intercellular spaces

During the past year, disease was induced in 4 of 8 volunteers by administering stool filtrate derived from an outbreak of gastroenteritis in England. Illnesses produced in our study were generally similar to those produced using this same material in England. Serum and stool specimens resulting from this study will now be examined further using methods previously applied to other infectious filtrates.

Attempts to produce illness in volunteers with a stool filtrate from a large outbreak of gastroenteritis in Morgantown, West Virginia, in 1971, failed; no illness occurred in eight volunteers given the filtrate.

IV. Epidemiology of Acute Infectious Nonbacterial Gastroenteritis - Epidemiologic characteristics of two separate outbreaks of nonbacterial gastroenteritis occurring at the same institution, Henryton State Hospital, Henryton, Maryland, were studied, and attempts were made to detect etiologic agents. Epidemiological patterns suggested either person to person spread, or a common source exposure with subsequent person to person transmission. Susceptibility to disease in both outbreaks among all age groups was noted, although younger residents were affected more frequently in the second outbreak. Some persons experiencing disease in the first outbreak were also ill during the second outbreak, thus indicating either short term immunity or antigenically distinct etiologic agents. Although no known enteropathogens could be demonstrated from either outbreak, the syndrome was induced in a human volunteer using a bacteria-free diarrheal stool filtrate derived from one of the outbreaks. The illness was subsequently reproduced by the administration of a similar filtrate prepared from stools from the ill volunteer. Experimentally induced disease was similar to naturally occurring illness in terms of incubation period, clinical signs and symptoms, and duration.

## Significance to Biomedical Research and the Program of the Institute

Acute infectious nonbacterial gastroenteritis is a common infectious disease which affects a broad segment of the population and was the second most common disease observed in a 10 year family study. Detection and characterization of a postulated etiologic agent present in a Norwalk-outbreak derived stool filtrate represent initial steps toward the ultimate goals of prevention and therapy. Information derived from volunteer studies and newly developed detection techniques for agents of acute infectious nonbacterial gastroenteritis will be useful in the development of serological assay systems and methods for *in vitro* cultivation of the agent(s).

## Proposed Course of the Project

Intensive study of etiologic agent(s) from outbreaks of acute infectious nonbacterial gastroenteritis will continue, using known infectious materials obtained through volunteer studies. Clinical materials from new outbreaks of gastroenteritis will be studied using laboratory methods which have proved

filled with an amorphous electron dense material. Brush border enzyme activities (alkaline phosphatase, trehalase, and sucrase) were decreased at the time of illness when compared with baseline and convalescent levels.

In addition to the above studies, jejunal IgA production was measured using biopsy material from the above group of volunteers. In the four men who became ill, there was a significant rise in IgA production in convalescent biopsies taken two weeks after illness, as compared with the changes in the three volunteers who did not become ill. The demonstrated rise in IgA production may represent a rise in secretory IgA in response to infection by the Norwalk agent, although it is not yet possible to determine how much of the IgA has specificity against the Norwalk agent.

II. Studies of Norwalk-derived and Other Filtrates in Culture Systems - No etiologic agents have been detected employing the tissue culture systems previously outlined. Current techniques in use are among the methods of choice for isolation of parvoviruses, and efforts are being continued to isolate viruses in these systems.

Previous studies indicated that the Norwalk agent may have been propagated in the in vitro system of human fetal intestinal organ culture. During the past year another volunteer study was completed, using an infectious inoculum of Norwalk agent which had been passaged five times through intestinal organ culture; the final inoculum had a calculated dilution of $10^{-13}$. Seven volunteers received this organ culture derived fluid and none became ill. Thus of eleven volunteers who received Norwalk material passaged in intestinal organ culture, in which at least a dilution of $10^{-13}$ was represented, only one has become ill.

Due to the inconclusiveness of these organ culture studies with the Norwalk agent, similar studies were carried out using "H" and "MC" filtrates. These original stool filtrates were passaged eleven times in intestinal organ culture. Rigorous safety tests failed to reveal the presence of any adventitious agents. In one study using "H"-derived organ culture fluids, (N.R. Blacklow, University Hospital, Boston, Mass.) 3 of 8 men receiving this inoculum developed illness. It was characterized by vomiting (1/3), diarrhea (3/3), abdominal cramps (3/3) fever $>99^4$ (1/3), and headache (1/3). In a subsequent study using the same inoculum, we found that none of six volunteers became ill. Thus, 3 of 14 men have been ill with gastroenteritis following administration of "H"-derived organ culture materials; it appears that the "H" agent may have grown in vitro but further studies are needed to demonstrate this conclusively. In another study using "MC"-derived organ culture fluids (N.R. Blacklow, University Hospital, Boston, Mass.), none of three volunteers became ill.

III. Transmission of Acute Gastroenteritis from Other Outbreaks - Previously disease has been induced in volunteers by the administration of stool filtrates derived from separate outbreaks in the following locations: a) Norwalk, Ohio, b) Bethesda, Maryland, c) Denver,Colorado d) Honolulu, Hawaii, e) Montgomery County, Maryland and f) Henryton, Maryland. Illness has occurred at varying frequencies although clinical features of experimental disease were generally similar.

successful in previous investigations. In vitro propagation of the agent(s) will be attempted further using a wide variety of tissue and organ culture systems. Further biophysical purification of the Norwalk particle and the subsequent development of a serologic assay will be major goals. The role of nonhuman primates as an animal model for acute infectious nonbacterial gastroenteritis will be pursued.

Honors and Awards: None

Publications:

Agus, S.G., Dolin, R., Wyatt, R.G., Tousimis, A.J., and Northrup, R.S.: Acute Infectious Nonbacterial Gastroenteritis: Intestinal Histopathology. Ann. Int. Med. (in press) 1973.

Blacklow, N.R. (Moderator), Discussants: Dolin, R., Fedson, D.B., DuPont, H., Northrup, R.S., Hornick, R.B., and Chanock, R.M.: Acute Infectious Nonbacterial Gastroenteritis: Etiology and Pathogenesis. Ann. Int. Med. 76: 993-1008, 1972.

Dolin, R., Blacklow, N.R., Wyatt, R.G., and Sereno, M.M. : Detection and Localization of Viruses in Human Fetal Intestinal Organ Cultures by Immunofluorescence. Infect. Immun. 6: 958-964, 1972.

Kapikian, A.Z., Wyatt, R.G., Dolin, R., Thornhill, T.S., Kalica, A.R., and Chanock, R.M.: Visualization by Immune Electron Microscopy of a 27-nm Particle Associated with Acute Infectious Nonbacterial Gastroenteritis. J. Virol. 10: 1075-1081, 1972.

Kapikian, A.Z., Gerin, J.L., Wyatt, R.G., Thornhill, T.S., and Chanock, R.M.: Density in Cesium Chloride of the 27-nm "8FIIa" Particle Associated with Acute Infectious Nonbacterial Gastroenteritis: Determination by Ultracentrifugation and Immune Electron Microscopy. Proc. Soc. Exp. Biol. Med. 142: 874-877, 1973.

Wyatt, R.G., Kalica, A.R., and Dolin, R.: Ultrastructural Study of Viral Replication in Human Fetal Intestinal Organ Culture. Arch. ges. Virusforschung (in press) 1973.

Serial No. NIAID 66I
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Natural History and Defective Nature of Infection with Adeno-Associated Viruses (AAV)

Previous Serial Number: Same

Principal Investigator: Dr. Raphael Dolin

Other Investigators: Dr. James Rose (Laboratory of Biology of Viruses)
Dr. Alan Rabson (Laboratory of Pathology)
Dr. M. David Hoggan (Laboratory of Viral Diseases)
Dr. Neil R. Blacklow (Boston Univ. School of Med.)

Project Description:

Details of this project were described in last year's annual report. This project has been discontinued.

Serial No. NIAID 69A
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Study of Nonbacterial Respiratory Disease
A. Children

Previous Serial Number: Same

Principal Investigator: Dr. Robert M. Chanock

Other Investigators: Dr. David Hodes
Dr. Albert Z. Kapikian

Cooperating Units: Dr. R.H. Parrott, Children's Hospital Research Foundation, Washington, D.C.

Contract Support:

| Contract Number | Project Off. (NIAID) | Contractor | Contractor's Project Dir. | Annual Funding Level |
|---|---|---|---|---|
| PH 43-68-1309 Part B | Dr. Chanock | Flow Labs | Dr. Gilden | $ 202,577 |
| NIH NIAID 71-2091 | Dr. Chanock | Children's Hosp. of D.C. | Dr. Parrott | $ 133,325 |

| Man Years: | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 24/12 | | | 24/12 |
| Professional: | 6/12 | | | 6/12 |
| Other: | 18/12 | | | 18/12 |

Project Description

Objectives: (1) To search for new agents responsible for respiratory disease in infants and children; (2) to delineate the epidemiology and pathogenesis of viruses which have been recently associated with respiratory disease; (3) to define the major determinants of host resistance to infection and illness; (4) to continue the surveillance of viruses previously established as playing a role in respiratory disease, i.e., the epidemiology and contribution to overall respiratory disease experience during infancy and childhood; (5) to evaluate experimental vaccines for safety, antigenicity, and effectiveness against naturally occurring respiratory tract disease.

Methods Employed:

Infants and children with respiratory disease and suitable control subjects without such disease are studied at the Children's Hospital, District of Columbia. The main emphasis is on the use of various tissue culture systems and artificial media for the recovery of viruses and mycoplasmas. All specimens from the patients are inoculated directly into tissue cultures and onto mycoplasma media without prior freezing and thawing, if feasible. In addition to specimens for isolation of viruses and mycoplasma, serial nasal washings are taken to study respiratory tract secretory antibody response.

After the various agents have been identified, their contribution to the different respiratory disease syndromes is estimated by comparing the recovery rate from such groups with that observed for healthy children free of respiratory symptoms. In addition, children with severe respiratory disease admitted to the hospital as well as comparable control subjects, are studied serologically for evidence of infection with the known respiratory viruses and mycoplasmas as well as any new agents which might emerge as potentially important pathogens during the course of the study.

Respiratory viruses which are attenuated by exposure to a chemical mutagen and which exhibit no virulence for adult volunteers are administered to children under carefully controlled conditions in the isolation unit of the Children's Hospital Clinical Research Center. As in other studies, parental consent is obtained in each instance. Vaccinees are observed closely for evidence of illness and specimens are taken for the study of virus excretion pattern, serum antibody response and local secretory antibody response.

Major Findings:

(1) Respiratory syncytial virus (RSV) - During the past 16 years the LID has engaged in a collaborative study of pediatric respiratory tract disease with Drs. Parrott, Kim and Brandt of Children's Hospital of D.C. From this study has emerged a relatively comprehensive understanding of the role of RSV in pediatric respiratory tract illness and of the importance of RSV in different respiratory disease syndromes. The data from the study were analyzed in detail during the past year and a number of significant correlations were observed. The importance of RSV as the major respiratory disease pathogen of infancy and early childhood was established beyond question during the 16 year period of study.

An epidemic of respiratory syncytial virus (RSV) occurred in the Washington, D.C. area during each of the 16 respiratory disease seasons between October 1957 and April 1973. There were alternating short (7 to 12 month) and long (13 to 16 month) intervals between peaks of successive epidemics. Each RSV epidemic lasted approximately 5 months and its peak was closely associated in time with a dramatic increase in the number of infants and young children who were hospitalized with lower respiratory tract disease, especially bronchiolitis and pneumonia. RSV epidemics primarily occurred in the late fall, winter or spring. Overall, February and March were the peak months for RSV disease. RSV was recovered from 27 percent of 1,179 inpatients with

bronchiolitis, 9 percent of 1,547 outpatients with acute respiratory tract disease but only 0.3 percent of 5,500 control subjects who were free of respiratory disease. Based on virus recovery and/or serum complement-fixation antibody response, RSV infection occurred in 43 percent of inpatients with bronchiolitis, 25 percent of inpatients with pneumonia and 23 percent of all inpatients with acute respiratory tract disease.

One factor which should be considered in assessing the impact of RSV in pediatric respiratory tract disease is the inefficiency of the CF and neutralization techniques for the detection of infection in young infants. This results in an underestimation of the importance of RSV in infants, the individuals most often made seriously ill by the virus.

One of the most remarkable features of the epidemiology of RSV in Washington, D.C. has been the consistent pattern of infection and disease. Other respiratory viruses have caused epidemics at irregular intervals, or have been endemic or have exhibited a mixed endemic-epidemic pattern, but RSV is the only respiratory viral pathogen which has produced a sizeable epidemic every year. Large annual variations in the impact of RSV on the pediatric population served by Children's Hospital of D.C. were not observed. The number and proportion of infants and children admitted to the hospital for RSV lower respiratory tract disease did not vary more than 2.7 fold. Also remarkable was the consistency with which RSV produced the same clinical pattern of respiratory tract disease, especially bronchiolitis and pneumonia, year after year. These illnesses were barometers of RSV infection in the community and when RSV was at its epidemic peak, hospitalization for bronchiolitis and pneumonia soared.

The behavior of RSV in the pediatric population of Washington, D.C. has been discerned primarily through the patterns of serious respiratory tract disease produced by this virus through a 15-1/2 year period. Although surveillance of seriously ill infants and children can serve as a barometer of the virus in the community, this type of study cannot, in itself, yield an estimate of the incidence of infection or risk of serious illness per infection. However, in unpublished seroepidemiologic studies we have found that approximately 1/2 of the infants tested were infected during their first RSV epidemic and almost all children were infected after living through 2 RSV epidemics. These observations suggest that the virus becomes widely disseminated during the epidemic and that the risk of infection for previously uninfected infants and young children is extremely high. If one couples this information with the estimated size of the pediatric population which was served by the Children's Hospital and the number of hospitalized patients with demonstrated RSV infection it is possible to derive a very crude estimate of the risk of serious RSV bronchiolitis, i.e. severe enough to require admission to the hospital, during primary infection. The crude estimate for the incidence of bronchiolitis requiring admission to the hospital per 1000 infants, 0-12 months of age, is 10 and for RSV bronchiolitis the estimate is 4. These estimates lead to a further estimate of 1 hospital admission for RSV bronchiolitis per 100 primary RSV infections during infancy. Of course the toll of RSV in infancy is considerably higher since for every hospital admission for RSV bronchiolitis

there are many infants who develop respiratory disease which is almost as serious as that seen in the individuals admitted to the hospital.

The occurrence of RSV infection and disease during 11 consecutive year epidemics was examined with respect to patient age, immunologic status, race and sex. The peak incidence of RSV bronchiolitis and pneumonia was observed at 2 months of age. Thereafter the incidence of these diseases decreased with increasing age, more rapidly for bronchiolitis then for pneumonia. In a previous study we had observed that the level of serum neutralizing antibody by age during infancy resembled the age distribution of RSV bronchiolitis. This similarity prompted us to suggest that serum antibody might participate in an immunopathological reaction in the lungs and that this reaction might contribute to the development and severity of bronchiolitis. During the past year the level of neutralizing antibody by age in the sera of control infants and in the acute phase sera of infants with RSV bronchiolitis or pneumonia was re-examined utilizing a larger collection of specimens than tested previously.

Each of the patients who had RSV bronchiolitis or pneumonia during the first 3 months of life possessed neutralizing antibody in his serum during the acute phase of illness. In most instances the level of this antibody, presumably of maternal origin, was moderately high. In contrast, 15 of 57 infants who developed RSV bronchiolitis at 4-7 months of age lacked detectable antibody in their acute phase serum. The latter finding casts some doubt upon the essential role of serum antibody in RSV bronchiolitis. Although the significance of serum antibody in the pathogenesis of disease may not be clear at this time, it is evident that relatively high levels of this type of antibody during the first 3-4 months of life do not protect the young infant from RSV bronchiolitis or pneumonia. At this point we are reluctant to dismiss completely the possibility that immunopathological factors play an important role in RSV bronchiolitis because of the strong suggestion in the past that an inactivated RSV vaccine had potentiated the response of vaccinees to RSV infection through an immunological mechanism.

The serum CF antibody response to RSV was impaired in infants 1-3 months of age. A delayed and/or decreased immunological response could contribute to the increased severity of RSV disease in early infancy. Recent studies of parainfluenza 1 (Sendai) virus in mice by Blandford and associates have demonstrated the occurrence of an early immunological response in the lungs which developed several days before serum antibody was detected. If this type of early pulmonary immunological response plays a role in resolution of infection, which is likely, a delay and/or decrease in response could lead to a more serious type of disease. One factor which might contribute to such an impaired response is immunological suppression produced by maternally derived serum antibody. This type of immunological suppression is known to affect the development of serum antibody, but it is not known whether local respiratory tract secretory antibody can be suppressed in a similar manner. It was found that the level of acute phase serum neutralizing antibody was inversely related to the height of the serum CF antibody response during convalescence suggesting that maternally derived antibody may have suppressed the immunologic response of some infants.

Stimulated by the observation that serum antibody was not protective in RSV disease evidence of a local immunological response was sought. This effort was successful and the development of neutralizing activity in nasal secretions was demonstrated following infection of a significant proportion of infants and young children with RSV disease. However, attempts to link local respiratory tract antibody with resistance and to define the level of local antibody which was protective have failed to date because the nasal secretions of young infants contain a non-antibody inhibitor of RSV. This inhibitor produces a low to moderate background of neutralizing activity in the secretions of infants and this makes it difficult to identify young subjects who lack specific, local respiratory tract antibody.

The clinical consequences of RSV infection in infancy did not appear to be influenced by race. The same proportion of males and females with respiratory tract disease yielded RSV; however, significantly more boys were admitted to the hospital for RSV pneumonia, bronchiolitis, croup and pharyngitis-bronchitis. During the 1960-1970 period the outbreak peaks of RSV (the months when the highest percentage of pediatric respiratory disease patients yielded RSV isolates) occurred in June 1960, January 1961, April 1962, February 1963, May 1964, December 1964, February 1966, January 1967, February 1968, February 1969 and April 1970. Since the outbreak peaks occurred in 6 different calendar months, data cumulated by month of the year would not show typical RSV epidemic wave, and could not adequately reflect the impact of RSV on pediatric patients at monthly intervals during a representative outbreak. We were interested in cumulating the experience from the 11 consecutive outbreaks since some aspects of the behavior of RSV might emerge only after analysis of a large number of patients or at least a larger number of patients than could be studied during one or two epidemics. Thus, data obtained at monthly intervals during the outbreaks were combined to plot a composite epidemic curve, which showed a "normal" distribution. Of the more than 1,000 respiratory disease patients who yielded an RSV isolate during the composite outbreak, 40.1 percent shed virus during the peak epidemic month and 8.24% shed virus during the 3 midepidemic months. During the peak month of the composite epidemic, RSV was recovered from 46.2% of all inpatients with bronchiolitis, from 33.6% of all inpatients with all forms of respiratory disease, and from 32.1% of all respiratory disease outpatients. Control subjects who were free of respiratory disease rarely yielded RSV. As indicated by virus recovery and/or the development of serum complement fixation antibody, 70.3% of bronchiolitis patients and 56.4% of all respiratory disease inpatients exhibited evidence of RSV infection during the peak epidemic month. A similar epidemic wave was seen in males as compared to females, and in black as compared to non-black children. More than half of all hospitalized patients who yielded an RSV isolate were less than 6 months of age. Among bronchiolitis patients, about half of those with RSV isolates were 3 months of age or younger.

Gardner et al have postulated that the essential process in RS bronchiolitis is in an allergic reaction which requires two infections with RSV, whereas RS pneumonia does not require a sensitizing RSV infection. The question of

one versus two infections in bronchiolitis is not merely of academic interest. Efforts to immunize infants with live attenuated RSV have begun, and are being continued with the expectation that protection rather than sensitization will result. Several findings, particularly our age distribution analyses, do not support the hypothesis that RSV bronchiolitis in infancy represents an "allergic" reaction to re-infection with the virus. In addition, the levels of IgE in the acute phase sera of 160 infants with RSV bronchiolitis were not elevated when compared to age matched controls. Finally, it appeared that RSV infection neither interfered with nor enhanced the occurrence of adenovirus infection in our study population.

Significance to Biomedical Research and the Program of the Institute:

Respiratory disease is the most common ailment of man, particularly during infancy and early childhood when many of the most serious expressions of such illness occur. Detailed knowledge of the agents responsible for respiratory tract disease and an understanding of their natural history are necessary before effective prevention can be achieved.

Proposed Course of Project:

It is planned that this study will continue for a period of years since a large segment of respiratory disease in infancy and childhood remains to be explained--approximately 40%. In addition, viruses responsible for respiratory illness vary in different times and in different localities; therefore, surveillance should continue in order to comprehend the larger picture. Efforts to increase our understanding of the pathogenesis of viral and mycoplasmal lower respiratory tract disease of infancy and childhood will continue.

Honors and Awards:

Director International Reference Laboratory for Respiratory Viruses other than Influenza, World Health Organization.

Director International Reference Laboratory for Mycoplasmas, World Health Organization.

Member International Subcommittee on Taxonomy of the Mycoplasmatales, International Committee on Nomenclature of Bacteria of the International Association of Microbiological Societies.

Served on Editorial Boards of American Journal of Epidemiology, Journal of Immunology, and Journal of Infectious Diseases.

Attended Meeting of Directors of W.H.O. Respiratory Virus and Enterovirus Reference Centres, London, April, 1973.

Recipient 1972 Gorgas Medal Award, San Antonio, Texas, December, 1972, Association of Military Surgeons.

Lecturer on Influenza, American College of Physicians, Baltimore, Maryland, March, 1973.

Elected American Pediatric Society, 1973.

Elected to Association of American Physicians, 1973.

Elected to National Academy of Sciences, April, 1973

Ricketts Award Lecture, University of Chicago, May, 1973.

Publications:

Kim, H.W., Arrobio, J.O., Brandt, C.D., Wright, P., Hodes, D., Chanock, R.M., and Parrott, R.H.: Safety and antigenicity of temperature sensitive (ts) mutants respiratory syncytial virus (RSV) in infants and children. Pediatrics. In press.

Parrott, R.H., Kim, H.W., Arrobio, J.O., Hodes, D.S., Murphy, B.R., Brandt, C.D., Camargo, E., and Chanock, R.M.: Epidemiology of Respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol. In press.

Brandt, C.D., Kim, H.W., Arrobio, J.O., Jeffries, B.C., Wood, S.C., Chanock, R.M., and Parrott, R.H.: Epidemiology of respiratory syncytial virus infection in Washington, D.C. III. Composite analysis of eleven consecutive yearly epidemics. Am. J. Epidemiol. In press.

Kim, H.W., Arrobio, J.O., Brandt, C.D., Jeffries, B.C., Pyles, G., Reid, J.L., Chanock, R.M., and Parrott, R.H.: Epidemiology of respiratory syncytial virus infection in Washington, D.C. I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. Am. J. Epidemiol. In press.

Serial No. NIAID 69C
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Etiology of Acute Respiratory Diseases in Adults

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: Mr. W. Lee Cline, Serology Unit, LID, NIAID
Dr. James MacLowry, Clinical Center, Clinical Pathology Microbiology Service
Dr. Wilmer Dockstader, Bacteriology Unit Environmental Services Branch

Contract Support:

| Contract Number | Project Off. (NIAID) | Contractor | Contractor's Proj. Dir. | Annual Funding Level |
|---|---|---|---|---|
| NIH-72-C-127CC | Dr. Kapikian | Microbiological Associates | Mr. M. Vincent | $ 6,555 |
| PH 43-68-1309 (Part B) | Dr. Chanock | Flow Labs. | Dr. Gilden | $202,577 |

| Man Years: | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 32/12 | | | 32/12 |
| Professional: | 1/12 | | | 1/12 |
| Other: | 31/12 | | | 31/12 |

Project Description:

Objectives: (1) To determine the role of known viruses and bacteria as etiologic agents of acute upper respiratory illnesses in adult populations; (2) To find and characterize new agents which may be human respiratory tract pathogens; (3) To investigate the sensitivity of new experimental cell culture systems for the isolation of rhinoviruses and the more recently described human coronaviruses.

Methods Employed:

A. National Institutes of Health (NIH) Volunteer Study - The current study began on September 9, 1965 and continues to the present. (An earlier similar study began in 1962 and was terminated in 1964). Employees of NIH with

respiratory illnesses volunteer a nasopharyngeal washing, a throat swab, and a blood specimen during the acute phase of a respiratory illness. An additional nasopharyngeal washing and blood specimen are obtained 3-4 weeks later when the volunteer has recovered from the illness. All of the acute nasopharyngeal washings are routinely inoculated into cell cultures of primary monkey kidney (MK), Hep-2, HDCS WI38, primary human embryonic kidney, human embryonic intestine MA177, and in addition, recently into BS-C-1 cells also. HDCS WI26 cultures and human aorta (AT-39) cell cultures were also used earlier in the study. In addition, the first 50 convalescent nasal washings and subsequently 1 of 4 such washings obtained through October 28, 1968, were tested in cell cultures also. Guinea pig erythrocytes are employed in hemadsorption tests in monkey kidney cell cultures for detection of myxoviruses whereas rat erythrocytes are employed similarly in BS-C-1 cell cultures for detection of OC43 related coronaviruses; rat red blood cells have been found to hemadsorb to OC43 virus-infected cell cultures, a finding reported in a previous annual report.

The throat swab is tested for beta (b-) hemolytic streptococci (in our laboratory), and in addition, beginning in March, 1969 for hemophilus influenzae, streptococci and other bacteria, by the Clinical Center Clinical Pathology Microbiology Service (Dr. MacLowry); if B-hemolytic streptococci are isolated they are grouped by the Microbiology Service Some of the nasal washings have also been tested for staphylococci with subsequent phage typing of recovered organisms (Dr. Dockstader).

In an attempt to find new agents, acute illness specimens which were negative for known viruses in the tissue cultures outlined above, are passaged three times in human embryonic tracheal organ cultures. The third passage harvest is clarified, concentrated, negatively stained with phosphotungstic acid and examined by electron microscopy for the presence of coronavirus-like or other particles. In addition, the harvest from the third passage is inoculated into the tissue cultures outlined above to determine if a cytopathogenic agent is recoverable. Recently, the additional technique of "immune electron microscopy" has been employed in an attempt to recover new agents, notably coronaviruses, from nasal washings which have failed to yield viral pathogens by tissue culture methods. A description of these studies is presented under Project 72A "New Techniques for Recovery of Respiratory Tract Viruses."

Major Findings:

From September 9, 1965 to March 31, 1973, a total of 708 volunteers enrolled in the NIH, LID Common Cold Study. From 0 to 35 entered the study each month; some were in the study more than once. At least one viral agent was recovered from 287 (41%) of the 708 study patients; only 2 patients yielded more than one viral agent. Beta hemolytic streptococci were recovered from 29 study patients (2 were group A, 6 group B, 5 group C, 3 group F, 7 group K, 2 non-groupable, and 4 not grouped). Three volunteers shed both a B-hemolytic streptococcus (not grouped) and a rhinovirus-like agent, and two shed a B-hemolytic streptococcus (one not grouped and the other non-groupable) and an adenovirus; ten shed both a B-hemolytic streptococcus belonging to groups

other than A and a viral agent. Two volunteers shed 2 viruses simultaneously--one both parainfluenza type 3 and an adenovirus, and the other both a rhinovirus and parainfluenza virus type 2.

192 (66%) of the 289 virus isolations were classified as rhinoviruses (all 192 were acid labile and chloroform stable) whereas 18 (6%) were identified as adenoviruses, 9 (3%) as herpes simplex, 11 (4%) as influenza A2, 7 (2%) influenza B, 6 (2%) parainfluenza type 1, 9 (3%) parainfluenza type 2, 4 parainfluenza type 3, 2 parainfluenza type 4, 6 respiratory syncytial, 23 (8%) coronaviruses (15 "229E-like," one each of OC strains OC37, 38, 43, 44, 48, 692, 700 and 1 untyped presumptive coronavirus tissue culture strain [859]) and 2 unclassified viruses.

As shown above, rhinoviruses were by far the most frequently recovered group of agents having been recovered from 27% of the study patients. However, the epidemiological pattern of rhinovirus infections has been noteworthy as the frequency of such infections has decreased during all 8 "winter seasons" of the study (December, January and February) in comparison to periods of higher prevalence during preceding "fall seasons" (September, October and November). For the 8 years combined, rhinoviruses have been recovered from 103 (42%) of the 248 study patients during the "fall periods" and from only 16 (8%) of 198 during the "winter periods." This difference in rhinovirus isolation rate is statistically significant at the .0001 level. During the "winter periods" other known respiratory virus agents were also recovered infrequently using conventional techniques.

Attempts to recover the etiologic agents of respiratory illnesses occurring during these fallow periods have been a major effort of this laboratory. These efforts have been fruitful with the use of two different culture techniques: human embryonic tracheal organ cultures and human embryonic intestine MA177 cell cultures. Details on the use of such cultures have been presented in previous annual reports and additional information on their use is presented in project number 72A. In brief, 12 of the 23 definite or presumed coronaviruses recovered from this study were isolated from nasal washings obtained in December, January, or February, 9 from washings obtained in March, and two in April. Although specimens obtained in the "winter" or "winter-early spring periods" have been tested most intensively, serologic studies from two such periods support the view that coronavirus infections with OC43 and 229E viruses may be important etiologic agents of acute upper respiratory illnesses in adults. In these serologic (CF) studies of paired sera presented in previous annual reports, coronaviruses were the predominant identifiable infecting organism associated with non-influenzal adult respiratory disease.

Paired sera obtained from patients admitted to the study during the 5 year period from September 1965 through August 1970 were tested for serologic (CF) evidence of infection utilizing numerous respiratory agent antigens. In addition, such sera were also tested by the HI technique for evidence of infection with OC43 virus. 110 (20%) of 541 patients developed significant CF and/or HI antibody rises; 10 of the 110 had simultaneous increases in antibody to two agents belonging to different antigenic groups, and one had

such increases to 3 such agents for a total of 122 rises. Of the 122 rises, 16 were to the parainfluenza viruses, 3 to adenovirus, 7 to RS virus, 17 to influenza A virus, 9 to influenza B virus, 31 to coronaviruses OC38 and/or OC43 and/or MHV-A59, 32 to coronavirus 229E, 4 to herpes simplex virus and 3 to M. pneumoniae. It is noteworthy that more than one-half (63 of 110) of the CF antibody increases were to the coronavirus antigens.

Further analysis of the data revealed that specimens from 541 study patients were tested by both virus isolation and serologic techniques for evidence of infection during the five-year period, September 1965 through August 1970; such evidence by either virus isolation or serologic (CF, or HI for OC43 virus) techniques or both was found in 289 (53%) of the 541 study patients. In addition, 2 patients had a group A beta hemolytic streptococcal infection. It was of interest that 62 (11%) of the 541 patients studied by virus isolation and serologic methods had serologic and/or viral isolation evidence of coronavirus infection--26 (5%) with 229E and/or "229E-like" viruses alone, 23 (4%) with members of the OC38 and/or OC43 or MHV-A59 virus group alone, 2 with other possible coronaviruses, 5 (1%) with more than one coronavirus only, 4 (1%) with a coronavirus and an agent of a different virus family, 2 with more than one coronavirus and an agent of a different virus family and one with a coronavirus and M. pneumoniae. Evidence of infection in a single individual with either 2 or all of the OC38, OC43, MHV-A59 group was tabulated as a single coronavirus infection.

Seroepidemiologic Studies of Coronavirus Infections in a College Population - In 1964 we described a pilot study of acute respiratory illnesses of University of Maryland students in which only 2 viruses (parainfluenza types 1 and 4) were recovered from 210 students; in addition, in an intensive serologic survey utilizing 21 CF antigens, serologic evidence of infection could be demonstrated in only 30 (25%) of 122 students from whom paired sera were available. Influenza B virus accounted for over one-half of these infections. The incidence of rhinovirus infections was not ascertained since methods for their study were not known and when such methods became known, the throat swab specimens were no longer available. With the more recent discovery of coronaviruses of human origin and their association with respiratory illnesses, we attempted to determine the incidence and prevalence of coronavirus infections in this college population by testing the paired sera still available from the previous study utilizing the CF (for 229E, OC38, OC43 and MHV-A59 viruses), HI (for OC43 virus) and the recently described neutralization-hemadsorption (for OC43 virus) techniques. The CF tests had been performed in 1969 whereas the HI and neutralization-hemadsorption tests were performed about 2-3 years later.

Paired sera from 110 of the 122 students whose sera were previously studied were available for testing by the CF technique for antibody to OC38, OC43, MHV-A59, and 229E viruses. 19 (17%) of the 110 students developed significant coronavirus CF antibody rises--12 to 229E and 7 to OC38 and/or OC43 and/or MHV-A59 viruses. The latter three strains were grouped together because of their previously described antigenic relationships and the frequent sharing of antibody responses to two or more of this group by individuals with corona-

virus infections. Paired sera from 93 of the students were also tested by the HI technique for OC43 virus antibody and no significant antibody increases were found; only 2 of the 7 paired sera demonstrating CF antibody rises were available for the HI antibody studies. It was of interest that 3 of 11 students with 229E virus antibody rises (one student was excluded because of anti-complementary activity in his acute serum) possessed 229E virus CF antibody in acute sera, and 2 of 7 with CF antibody rises to one or more of the OC38 virus group had such antibody to at least one of the antigens to which a rise had occurred. It was also noteworthy that 12 of the 19 students with serologic evidence of coronavirus infection had either an afebrile rhinitis or an upper respiratory illness, 2 an afebrile non-exudative pharyngitis, 2 an afebrile exudative pharyngitis, 2 an influenza-like illness and 1 a pneumonitis.

The prevalence of antibody to these viruses varied and depended upon the technique used for its measurement. 26% of the students with available sera had CF antibody titers of >1:8 to 229E virus, 19% to OC38 virus, 36% to OC43 virus, and 11% to MHV-A59. (In the CF test an acute serum was tested only if the convalescent serum had a titer of >1:8 in the screening test; in such an instance the acute serum titer was included in the prevalence data tabulation. If the acute serum titered 1:4, it was tabulated at <1:8; if the convalescent serum titered <1:8, this value was included in the prevalence data tabulation.) Prevalence of OC43 virus antibody was also studied by the HI and neutralization-hemadsorption techniques using acute phase sera. By HI technique, 84% of the students had titers of >1:8, whereas by the neutralization-hemadsorption test, all had titers of >1:8; it was noteworthy that 90% of the neutralization antibody titers were >1:32. A comparison of HI and neutralizing antibody titers was also made; identical titers or titers varying only two-fold were found for one-third of the sera whereas in the remainder the neutralizing antibody titers were at least 4 fold greater than those determined by the HI technique. 229E virus neutralizing antibody titers in acute phase sera were also determined and 39% of the students had titers of >1:8, the 229E titers were not nearly as high as those observed for OC43 virus, as only 5% were >1:32. These prevalence data indicate that infection with OC43 or OC43-related agents is very common whereas 229E virus infection occurs less frequently. It appears from this and previously reported studies that OC43 virus infections in adults occur predominantly as reinfections, a pattern similar to that observed with both RS and parainfluenza 3 virus infections in adults. Definitive assignment of the relative importance of the coronaviruses as etiologic agents of acute respiratory diseases must await the development of suitable sensitive methods for their cultivation.

Significance to Biomedical Research and the Program of the Institute:

These studies are aimed at evaluating the role of known viruses and bacteria as etiologic agents of adult respiratory diseases and at finding new agents which cause these illnesses. It was noteworthy that with the use of both virus isolation and serologic techniques over 50% of the illnesses studied among NIH, LID Common Cold Study volunteers were found to be associated with a viral agent. In addition, the recovery of coronaviruses during certain

"winter" and "winter-early spring" periods when non-influenzal respiratory illnesses were prevalent but virus isolation rates paradoxically low was noteworthy. It appears that with the use of new techniques such as organ cultures and experimental cell cultures new agents can be recovered during such previously fallow periods. Elucidation of the etiology of the majority of upper respiratory illnesses during repeated respiratory disease seasons must be accomplished before attempts at prevention of such illnesses can be fruitful.

Proposed Course of Project:

These studies which attempt to find new agents associated with adult respiratory illnesses will receive major emphasis in this laboratory as recent efforts to recover such agents by using human embryonic organ cultures and experimental cell cultures have been highly rewarding. In addition, the role of bacteria alone or in association with viruses in the etiology of respiratory disease will be studied more extensively.

Honors and Awards: None

Publications: None

Serial No. NIAID 70A
1. Infectious Diseases
2. Respiratory Viruses
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Project Title: Laboratory Studies of Myxoviruses, Paramyxoviruses and Respiratory Syncytial Virus

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Project Description:

Objectives: (1) To produce conditional lethal temperature-sensitive (ts) mutants of myxoviruses, paramyxoviruses and RS virus for possible use in live attenuated vaccines; (2) to study the genetic basis for attenuation shown by temperature sensitive mutants; (3) to define the relative importance of the various host defense mechanisms in resistance to myxovirus and paramyxovirus infection and disease.

Methods Employed:

A. Influenza - Influenza A virus suspensions were initially prepared in bovine kidney (BK) tissue culture. Mutagenesis of influenza A/HK/45/68 (H3N2) was accomplished by growth of this virus in BK roller tube cultures in the presence of medium containing $10^{2.5}$ M 5-fluorouracil. High titered suspensions of virus for use in volunteer studies and for genetic characterization of temperature sensitive mutants were prepared by growing the bovine kidney passaged virus in the allantoic sac of either avian leukosis free eggs (for

volunteer studies) or of regular embryonated eggs (for strains to be used for laboratory characterization).

Genetic analysis of temperature sensitive mutants was accomplished in the following manner. In the plate complementation-recombination technique allantoic fluid suspensions of different mutant pairs were mixed, and inoculated onto monolayers of BK or rhesus monkey kidney (RMK) cells in petri dishes according to the usual plaque technique for the quantitation of influenza virus. During the year this technique was modified to include pre-incubation of the 2 mutants of 4°C for 18 hours prior to inoculation onto the assay monolayer culture. This type of pre-incubation has been shown by Hirst to increase the frequency of genetic interaction between mutants. The cultures were incubated at a temperature restrictive for plaque formation for each of the viruses individually. If plaques did develop the viruses inoculated were assumed to possess genetically distinct lesions, one virus providing for its partner in the cross the gene product (or products) which was defective in the other and thereby enabling plaque formation to occur. Plaques which developed at restrictive temperature contained wild type virus and thus this method of genetic evaluation assayed the occurrence of recombination.

Major Findings:

A. Respiratory Syncytial Virus - Research on respiratory syncytial virus (RSV) has proceeded along several lines; (1) further characterization of existing ts mutants of the virus; (2) production of new mutants; (3) studies of the mechanism of pathogenesis of the virus and of the mechanisms of host resistance; (4) examination of basic biologic properties of the virus and of its infectious process in cells.

(1) Characterization of existing ts mutants - During the past year several problems alluded to in the previous report have been solved and refinements of previous studies devised. After plaque isolation procedures were employed, a stable clonal population of the ts-2 mutant was obtained. This clonal population failed to produce plaques at restrictive temperature and, at permissive temperature reproducibility exhibited the altered plaque morphology which had seemingly been lost in other populations of the mutant. Using this clone, the plaque complementation studies described in the 1971-1972 annual report were completed, and the mutants were grouped into three complementation groups. One group was represented by the 5-FU induced mutants, ts-1, ts-3, ts-4, ts-5 and ts-6. A second group was represented by the ethyl-methanesulfonate (EMS) induced mutant, ts-7. These two groups had already been elucidated by earlier growth complementation experiments and were further confirmed by plaque complementation. Ts-2, which exhibited considerable growth at restrictive temperature (leakiness) as well as altered plaque morphology, was demonstrated to belong to a third complementation group. Cells doubly infected with ts-2 and with a mutant belonging to either of the other complementation groups, produced plaques when incubated at restrictive temperature. In contrast, cells singly infected with any of the mutants and cells doubly infected with mutants belonging to the same complementation group, produced no plaques when incubated

at the same restrictive temperature. The leakiness of ts-2 had previously prevented its characterization in growth complementation experiments.

Further refinements were undertaken in the step-up experiments described in last year's report, and, in addition, step-down experiments were performed. In the step-up experiments, cell cultures infected with each of the mutants were incubated at permissive temperature for either 6, 12 or 18 hours. At the end of each of these time periods, the cultures were shifted to a restrictive temperature (the high restrictive temperature of 39°C was used to minimize leakiness) and incubation continued until the 24 hour time point. Cultures were then harvested and the virus yield determined by the plaque technique at permissive temperature. In such experiments, cultures infected with mutants with lesions affecting functions operative before the step-up should not show any decrease in virus yield compared with infected cultures incubated for the full 24 hours at permissive temperature. In the step-down experiments, by contrast, infected cultures were initially incubated at restrictive temperature and then shifted to permissive temperature at 6, 12 or 18 hours. In these experiments the virus yield of cultures infected with mutants defective in late viral functions should be unaffected. The results of these experiments were reproducible and indicated that ts-1, ts-3, ts-4, ts-5 and ts-6 mutants, which belong to a single complementation group, were all defective in a function operative between hours 12 and 18 in the viral growth cycle. In contrast, ts-7, which formed a separate complementation group, was deficient in an earlier function operative between 6 and 12 hours. Thus the step-up and step-down experiments supported the classification of mutants arrived at through complementation studies. Ts-2 showed too much leakage in the step-up and step-down experiments to allow for accurate determination of defective segment of its growth cycle.

Earlier studies in hamsters and in adult volunteers gave hope that ts-1 would be an acceptable strain for use as a live vaccine to be given to young infants. Unfortunately, clinical trials conducted during the past several years at the Children's Hospital of the District of Columbia revealed two problems with ts-1. First, although the virus was almost completely attenuated for seronegative infants, a small amount of residual virulence was detected. Second, isolates from some of the children vaccinated with ts-1 exhibited evidence of altered temperature sensitivity, indicating that ts-1 was not completely stable genetically in young children.

In regard to this second question, we undertook to define the nature of the genetic instability of ts-1. Using plaque purification techniques, clones were isolated from the progeny populations obtained from vaccinees which gave evidence of altered temperature sensitivity. Some of these purified virus clones formed plaques with equal facility at 32°C, the permissive temperature, and at 37°C, 38°C and 39°C, restrictive temperatures for ts-1. These clones thus appeared to have reverted to a wild phenotype. Other purified clones had acquired a more limited ability to form plaques at restrictive temperatures: plaque formation was either strictly limited to the lower restrictive temperatures or decreased in efficiency at the higher restrictive temperatures. Such clones were clearly intermediate in their temperature sensitivity between ts-1 and wild type RSV. It is likely that such clones probably arose as the result of suppressor

mutation in the ts-1 genome. In the language of classical genetics, a suppressor mutation is a second mutation which serves to correct, or partially correct, the defective function of a mutant. Suppressor mutations have been studied extensively in bacterial and phage genetics, but have not been noted previously in animal viruses.

(2) Production of new mutants from ts-1 - The difficulties encountered with ts-1 made it desirable to develop alternative candidate vaccine strains. As genetic stability was a primary concern, efforts were directed at determining whether any of the other existing mutants was more stable than ts-1. To this end, a "stress test" was devised to simulate in vitro the growth of virus in volunteers. Each of the ts mutants was incubated in HeLa cell cultures at 37°C for an extended period of time (4-6 days). At the end of the period, the cultures were harvested and the progeny virus tested for evidence of loss of temperature sensitivity by performing plaque assays at both permissive and restrictive temperatures. Ts-2, because of its leakiness, was not considered a suitable vaccine candidate. Ts-3, 4, 5, 6 and 7 all showed evidence of genetic alteration after being subjected to an in vitro stress test. It was thus decided that new mutants would have to be derived.

It is generally accepted that mutants having multiple lesions are far more likely to be stable genetically than are mutants having a single lesion. Thus we attempted to introduce an additional lesion(s) into ts-1 by further treatment of this strain with a second mutagenizing agent. EMS was chosen as the agent since it had previously induced a lesion in ts-7 which proved distinct from the lesion of ts-1.

A population of ts-1 was exposed to EMS for 105 minutes, resulting in a 1000-fold decrease in virus titer. The surviving virus was inoculated into bottles of BK tissue culture and clonal populations of the progeny were isolated by the plaque technique. Three hundred viable clones were isolated in this fashion. The isolated clones were then analyzed to determine if any of them was distinct from ts-1. This task was made more difficult by the lack of identifiable ts-1 markers other than temperature sensitivity, but was attempted in two different ways: (1) since stress tests of ts-1 either yielded progeny which produced plaques at restrictive temperature or revealed moderately high titers of virus yield at restrictive temperature, clones were sought which on stress testing, showed neither of these two properties; and (2) since ts-1 produces protein as detected by indirect immunofluorescence at restrictive temperature, clones were sought which did not produce viral protein at 39°C. Each of the 300 isolated clones was subjected to both stress testing and indirect immunofluorescent evaluation. Despite numerous false positive results none of the clones proved distinct from the parent ts-1 mutant on repeated testing. Our failure to produce an additional recognizeable mutation with EMS was disappointing especially in light of the many false leads and the ease with which the ts-7 mutation had been produced. It is possible that passage of the EMS exposed virus in BK cells may have provided a selective advantage to the genetically unmodified ts-1. A mutant with multiple lesions may have been at disadvantage as regards growth so that its progeny may have formed only an insignificant portion of the BK passaged virus. We are currently

attempting to repeat the EMS treatment of ts-1 with direct plaque isolation of the surviving virions. Doubtless, the isolation of clones in this fashion will be more diffiult, but the possibility of finding double mutants should be improved by decreasing the possibility of overgrowth by the healthier single mutant.

(3) Studies on the pathogenesis of RSV disease - One of the most serious obstacles to the elucidation of the mechanism of RSV pathogenesis in human infants is the lack of a suitable animal model. This circumstance is particularly frustrating because the mechanism of pathogenesis is apparently quite complex. The roles of the different immunologic mechanisms in protection from RSV disease are unclear, and there is a distinct possibility that some of the pulmonary damage itself may be immunologic. In the last annual report, we recorded attempts to produce a model of RSV bronchiolitis in the immunologically crippled hamster. Despite treatments with X-irradiation and cyclophosphamide, we failed to produce pulmonary lesions when animals were challenged with wild type RSV, although these treatments did seem to retard the clearance of virus from the hamster respiratory tract and to delay serum antibody response. In an attempt to produce immunologically mediated pulmonary injury we administered a hyperimmune hamster anti-RSV serum to a group of hamsters in a second series of experiments. When these animals were challenged with RSV, however, their lungs showed no pathologic lesions. Hyperimmune serum treatment did seem to facilitate clearance of virus from the hamster respiratory tract, but this conclusion is still tentative since the appropriate reconstruction experiments to rule out the possibility of in vitro neutralization are still in progress.

Recent reports from Russia have given hope that the 10 day old wild cotton rat may develop pulmonary lesions when challenged with RSV similar to those seen in infantile bronchiolitis. Unfortunately, this species is no longer readily available for laboratory work, and efforts are currently being made to obtain a source of these animals. Attempts to infect 10 day old albino laboratory rats with RSV were unsuccessful.

Despite the lack of an animal model for RSV induced disease, it is still possible to elucidate possible mechanisms of virus-host interactions by means of in vitro techniques. For example, work done in the laboratory several years ago (Dr. Helen Coates) indicated that complement had an enhancing effect on the ability of RSV-neutralizing antibody to prevent production of plaques by the virus. In such tests, antibody and virus were reacted together in vitro for one hour, and the mixture was subsequently inoculated onto tissue culture monolayers for quantitation of virus plaque forming units (PFU).

Although it is quite likely that this complement-enhanced mechanism of viral neutralization is of significance in the infected host, it should be pointed out that there are some artificial aspects of the system. In an infected cell monolayer, only a small percentage (c. 10%) of the total virus yield is found in the extracellular fluid. The bulk of virus formed remains cell-associated, and cell-to-cell transmission seems to be accomplished mainly by the fusion of contiguous cell membranes (syncytium formation). It was therefore

of interest to determine the effect of antibody on cells already infected with RSV. To this end, layers of Hep-2 cells were infected with RSV and treated with either antibody alone or with a combination of antibody and complement during the following periods of time: (1) during the first 2 hours after inoculation (adsorption period); (2) between 2 and 14h° after inoculation (latent period); (3) between 14h° and 24h° after inoculation (during period of most rapid viral growth); and (4) between 24h° and 48h° after inoculation (throughout second cycle of infection). The number of plaques developing in each of these cultures was determined at 72h°. Using these techniques, it was shown that RSV plaque formation could be inhibited by treatment of the infected monolayer with antibody, in the absence of complement, during any portion of the growth cycle after adsorption. In contrast antibody-mediated neutralization of cell-free virus required complement. Further, lysis of infected cells did not seem to be involved since experiments in which the infected monolayers were trypsinized and the cells inoculated into new tissue culture wells revealed that the antibody treatment did not diminish the number of infectious centers. It is possible that antibody might operate in a number of different ways under these conditions. For example, antibody, without the help of complement, might prevent recruitment of cells into a syncytium and thus inhibit plaque formation even though virus remained viable in the initially infected cells.

(4) Studies on the basic biologic properties of RSV and its infectious process - Examinations of the basic biologic properties of RSV are of importance to the laboratory's program for several reasons: first, the purification of viral products is essential for the development of sensitive and specific tests, such as radioimmunoprecipitation (RIP) for the detection of anti-RSV antibody in the nasal secretions of infants. With the currently available techniques, non-specific inhibitors in these secretions make study of secretory antibody extremely difficult, and this important parameter of immunity cannot properly be evaluated. Second, more refined studies of RSV biochemistry should allow for better classification of the ts mutants and for clearer elucidation of viral functions. Third, such studies may clarify the taxomic status of the virus. Finally, it may be possible to pinpoint steps in the viral growth cycle which may ultimately prove vulnerable to chemotherapy.

Basic studies on RSV have been greatly hampered by the difficulty in preparing high-titered, purified stocks of virus. We therefore attempted to study specific viral products which accumulate in the RSV-infected cell. During the past year, we undertook to isolate a RSV specific RNA from RSV-infected cells. This aim has been achieved by means of adding tritiated uridine of high specific activity to infected and uninfected HeLa cells pre-treated with actinomycin D to suppress host cell DNA-dependent RNA synthesis. By extracting such cells with hot phenol, the gradual emergence of RSV-specific RNA during the course of a 24h° growth cycle was delineated. When this RNA was analyzed in sucrose density gradients the principle species appeared to peak at 9-10S, with considerable heterodisperse RNA being present. The overall pattern resembled that seen in cells infected with paramyxoviruses, but the heavier species characteristic of paramyxovirus virion RNA was not detected. There is evidence that much of the lighter RNA extracted from cells

infected with a number of paramyxoviruses is single-stranded viral messenger RNA. Current results do not allow such a conclusion regarding the RSV-specific RNA, but at least 80% of it is sensitive to digestion of N. Crassa ribonuclease. This would argue that it is single-stranded. The nature of the ribonuclease-resistant fraction is currently being investigated and preparations are being made to initiate studies of the RNA patterns in cells infected with each of the different ts mutants.

B. Influenza Virus -

(1) Isolation and characterization of temperature sensitive (ts) mutants and recombinant viruses of influenza A (H3N2) Hong Kong 1968 - As previously described, four temperature sensitive influenza A recombinant viruses with the surface antigens of the Hong Kong virus (H3N2) were derived by mixedly infecting BK tissue culture with wild type Hong Kong virus (H3N2) and a temperature sensitive mutant of influenza A/Great Lakes/65 (H2N2) virus. These recombinant ts viruses were designated: (a) influenza A (H3N2)-ts-1[A] virus (37°C shutoff temperature); (b) influenza A (H3N2)-ts-1[E] virus (38°C shutoff temperature); (c) influenza A (H3N2)-ts-1[H] virus (39°C shutoff temperature); and (d) influenza A (H3N2)-ts-2[C] virus (39°C shutoff temperature). The four recombinant viruses were shown by genetic analysis to possess lesions in different functional portions of the influenza A virus genome, and each has been administered to seronegative volunteers with valuable information obtained concerning the relationship between temperature sensitivity (a measure of defectiveness) and attenuation of these viruses for man. Further it was shown that a lesion in any one of 4 distinct cistrons was capable of disabling the virus and rendering it attenuated for man. However, since influenza A viruses possess from 7 to 10 pieces of RNA which are believed to represent functional portions of the influenza A viral genome, it is clear that we have explored the effect of ts mutation on approximately only one-half of the influenza genome. We have therefore produced more ts mutants in order to: (1) derive a series of recombinants which have a single lesion or cluster of lesions on one of the functional portions of the influenza A virus genome with the aim of producing ts viruses which are specific for each of the viral genes; (2) derive separate mutants which have a ts defect in the same segment of the influenza A virus genome but which differ as regards temperature sensitivity; (3) further evaluate ts viruses in sero-negative volunteers to explore more fully the relationship between attenuation, site of genetic lesion and level of temperature sensitivity.

A wild type suspension of influenza A/Hong Kong/45/68 (H3N2) virus was mutagenized directly by growing virus in $10^{2.5}$ M 5-fluorouracil. From approximately 1000 plaques studied, 5 different clones of ts virus were isolated which were genetically stable after cloning and growth to high titer in the allantoic cavity of embryonated eggs. One virus had a shutoff temperature of 37°C; three shutoff at 38°C, while another shutoff at 39°C. These viruses were subjected to genetic analysis by the plate complementation-recombination method. The ts-304 (H3N2) virus (38°C) was found not to share a lesion with the other 4 mutants or with the 4 distinct ts recombinants of Hong Kong virus. Thus, we have produced a ts mutant (304) which appears to have a single lesion which differs from those of the 4 distinct ts recombinants.

This means that we now have ts mutants which represent 5 cistrons of the influenza A virus genome. The other 4 ts mutants shared ts lesions with one or more of their group as well as with the H3N2-ts recombinants. Several of these viruses appeared to possess ts lesions in more than one segment of the genome. Nonetheless these 4 mutants may be very valuable to the program in that they may serve as the source of single lesions which can be transferred to other viruses by the process of recombination. It should be remembered that the ts-1 mutant of H2N2 served as the source of 3 distinct, stable, presumably single lesion, ts recombinants following mating with an H3N2 virus.

(2) ts recombinants of influenza A (HON1) - We have selected an influenza A/Ann Arbor/Marton/43 (HON1) virus as the wild type virus for recombination with our H3N2 ts viruses for the following reasons: (1) the HON1 virus differs from the H3N2 virus in both surface antigens thereby permitting exploration of the relationship of the ts lesion to the two surface antigens; (2) an HON1 wild type virus was available which had a history of passage only in embryonated eggs, a substrate which is suitable for growth of virus to be used for volunteer studies; (3) all individuals born after the HON1 virus disappeared from circulation in 1947 should be seronegative for the HO hemagglutinin and therefore could serve as volunteers. Importantly, the mating of a ts H3N2 vaccine virus with the HON1 virus might represent a model for attenuation of the next expected pandemic influenza A virus (H4N3). Because the influenza A (H3N2) ts-1[E] virus shows promise as a live influenza A vaccine strain, a mating of this virus and the HON1 virus was undertaken in BK cell culture. Over three hundred clones of virus with the HO surface antigen derived from the above cross were examined and 10 percent were found to be temperature sensitive. Thus, ts viruses with the HO surface antigen were isolated with high frequency. The temperature sensitivity of one clone of HO-ts-1[E] virus (6B1) was examined in detail and was found to be identical to that of the donor parent influenza A (H3N2)-ts-1[E] virus, i.e., 38°C shut-off temperature. This suggests that the degree of temperature sensitivity was determined by the ts-1[E] defect.

The capacity of the HO-ts-1[E] virus to grow in the lungs of mice was studied in detail. The growth capacity of the H3N2 wild type (wt), H3N2-ts-1 [E] and HON1 wt viruses was also assessed in the same experiment. The growth of the H3N2-ts-1[E] virus and the HO ts-1[E] virus was reduced from 10 to 100-fold over that of their respective wt viruses. Thus, in vitro temperature sensitivity and restriction of replication in mouse lung appeared to be phenotypic manifestations of the same genetic defect.

Since hemagglutin and neuraminidase surface glycoproteins segregate independently the HO-ts-1[E] recombinant viruses could be either HON1-ts-1[E] or HON2-ts-1[E]. Thirty clones of virus with the HO-ts-1[E] phenotype have been isolated from the cross of H3N2-ts-1[E] X HON1 wild type virus using antiserum monospecific for the H3 hemagglutinin for neutralization of the H3 parent and H3 recombinants. These clones are being analyzed for the presence of either the N1 or N2 neuraminidase by Dr. J. Kasel. Preliminary evidence indicates that clones of HO ts-1[E] virus can possess either the N1 or the N2

neuraminidase. This suggests that the ts-1[E] lesion is not present in the genetic material that codes for the neuraminidase because the ts defect segregates independently of the neuraminidase antigen. Previously we had shown that the ts-1[E] lesion was not in the gene which codes for the hemagglutinin.

Like the parent H3N2-ts-1[E] virus, the HO-ts-1[E] virus clones were genetically stable in vitro. In order for a virus to be genetically stable after growth to high titer ($10^{7.2}TCID_{50}/ml$), it is likely that the genome had undergone multiple ts mutations. These putative multiple lesions could be on the same segment of RNA or on different segments of RNA. If the latter were the case, it should be possible to isolate clones of HON1 ts virus which will exhibit genetic interaction (i.e., recombination) with each other as was observed with the H3N2-ts-1[A], H3N2-ts-1[E], and H3N2-ts-1[H] recombinant viruses derived from the H2N2-ts-1 virus. Seventeen clones of recombinant virus HON1-ts-1[E] (3x cloned) have been prepared in sufficient quantity and titer to begin extensive cross recombination tests to determine if the lesion or lesions of the H3N2-ts-1[E] virus are limited to one segment of its genome.

(3) ts recombinants of influenza A (H3N2) London 1972 virus - The occurrence in 1972 of antigenic variation in the coat of the Hong Kong 1968 strain of influenza A provided an opportunity to study the feasibility of transferring both temperature sensitivity and attenuation of the influenza A/Hong Kong/68 (H3N2) ts-1[E] virus to a new strain within the H3N2 subtype. Some cross protection against influenza A/Eng/42/72 (H3N2-72) virus appears to be afforded by inactivated vaccine containing the Hong Kong-68 virus. However, it is clear that this virus is sufficiently different so that optimal protection requires a vaccine which contains antigens that closely match those of the new virus. We found in a retrospective study that only 50% of the volunteers infected with either the Hong Kong wild type or ts-1[E] virus developed antibody to the London (H3N2) 1972 virus, whereas 90% developed antibody to the (H3N2) 68 virus. In addition, further testing of the H3N2-68-ts-1[E] virus in adult volunteers has become increasingly difficult because most of the population now possesses serum antibody to this virus. For these reasons, we have isolated ts viruses with the hemagglutinin of the A/England/42/72 virus from a cross between our influenza A/Hong Kong/68-ts-1[E] virus and wild type influenza A/England/42/72 virus. The progeny from this cross were mixed with an appropriate antiserum monospecific for the 1968 hemagglutinin at a dilution that suppressed growth of the 1968 virus but permitted plaque formation by the 1972 virus. Plaques which developed at the permissive temperature (33°C) were picked and tested for temperature sensitivity. Of 30 plaques picked, 9 (30%) were temperature sensitive. This high frequency of recombination, reminiscent of the results with the HON1 virus described above, demonstrated that temperature sensitive mutants with the desired surface antigens could be produced rapidly and readily in a single step. In vitro studies showed that the H3N2-68-ts-1[E] and H3N2-72-ts-1[E] viruses exhibited the same degree of temperature sensitivity as regards plaque formation in rhesus monkey and calf kidney monolayer cultures. This result again demonstrates that the level of temperature sensitivity exhibited by the parent and recombin-

ant ts viruses is determined by the ts lesion and not by other factors present in the recipient virus.

Studies in mice showed that the recombinant 1972-ts-virus exhibited restricted replication in mouse pulmonary tissue, like the parent 1968 ts-1[E] virus. Suspensions of virus for identification of neuraminidase antigen are being prepared. In addition, suspensions of wild type 1972 (H3N2) virus and 1972 (H3N2)-ts-1[E] virus have been prepared for evaluation in volunteers this summer and fall.

(4) ts mutants of influenza B virus - Influenza B virus is a respiratory pathogen which infects all age groups and is associated with excess mortality in some years which is comparable to that caused by influenza A virus. We have therefore begun to develop a live virus vaccine using the methods employed with influenza A virus. We have mutagenized influenza B/Maryland/1959 virus by growing the virus in the presence of 5-fluorouracil. Approximately 400 plaques have been characterized for temperature sensitivity and 10 appear to have genetically stable ts defects. These 10 ts viruses are currently being cloned. The temperature sensitivity of these viruses ranged from 37° to 39°C. Large suspensions have been prepared for genetic analysis, animal studies, and recombination with current influenza B viruses.

Efforts were originally planned for recombination of influenza B/59-ts viruses with influenza B/71 wild type virus with the production of influenza B/71-ts recombinant viruses. However, influenza B viruses isolated during 1973 in Asia and Australia have shown major antigenic change in the hemagglutinin when compared to the recently prevalent influenza B viruses, i.e., influenza B/Mass/71. It is likely that these new influenza B viruses will become the prevalent influenza B strains so our efforts will be directed toward production of ts mutants of this virus by recombination with the influenza B/59-ts viruses. These temperature sensitive influenza B/73 viruses will be evaluated in volunteers.

(5) Host cell factors which affect genetic interaction of temperature sensitive mutants of influenza A virus - In the basic assay used to determine whether two ts mutants share lesions in the same piece of the RNA genome monolayer cultures are inoculated with the 2 viruses and incubation is carried out at a restrictive temperature. Subsequently the number of plaques which develop after mixed infection is compared with the number of plaques produced by either virus alone; the latter value is usually zero. Plaques which develop at restrictive temperature after mixed infection can be attributed to the fact that the various segments of the influenza genome can assort independently and that a mixed infection in which two ts mutants do not share lesions there is, in the infected cell, a complete, nondefective set of the 7-10 RNA pieces.

The multiplicity of double infection can be estimated by ascertaining the number of plaques formed by each of the mutants at the nonrestrictive temperature. Using these values the number of doubly infected cells can be calculated and a ratio can then be formed between the number of plaques observed and the number expected at the restrictive temperature if recombination following

mixed infection were 100% efficient. When the data were compared among tests in which the same pair of mutants but different lots of rhesus monkey kidney cell were employed, two points emerged: (1) there were certain lots of cells in which essentially no recombination occurred between mutants which had been shown in other tests to be genetically distinct; (ii) there were certain pairs of mutants which consistently and readily underwent genetic reassortment (e.g. ts-2[C] and ts-304), while there were other extremely fastidious mutants (most notably ts-1[H]) which replicated and recombined only in lots of cells in which recombination of other mutant pairs occurred with very high frequency. The variability just described points clearly to a cell factor which could be inate to the cell or possibly it could be the result of undetected adventitious agents present in the cell.

In any case host cell variability is an important factor which might be recognized and hopefully some day its mechanism will be elucidated. This phenomenon has one practical implication. When evidence of genetic recombination is observed in a well controlled experiment, the result can be interpreted with confidence. However, failure to detect evidence of genetic interaction cannot be interpreted with the same degree of confidence; in some instances it may be only a manifestation of the host cell factor which affects genetic reassortment of influenza A virus RNA.

A method to minimize some of the variability in reassortment observed in the case of fastidious mutants was suggested to us by Dr. George Hirst. This procedure consists in mixing undiluted suspensions of the two mutants and incubating them for 18 hours at 4°C prior to dilution and plating. It is hypothesized that the incubation of 4°C facilitates the physical clumping together of virions of the 2 types and thus insures that a greater proportion of the cells will be doubly infected. Two points in relation to this method should be noted: (1) the enhancement is seen mostly with the fastidious mutants and (2) reassortment is not enhanced in cells in which genetic reassortment is inefficient or non-existent with mutant pairs which ordinarily show high frequency of recombination.

C. Parainfluenza 1 -
A temperature sensitive mutant (ts-4) had previously been produced with 5-fluorouracil and shown to be genetically stable in tissue cultures. Unfortunately, infection of hamsters resulted in the emergence of revertants in the lungs with prompt growth predominance of the wild type revertants. Attempts were then made to stabilize the conditional lethality of the mutant by introducing additional temperature sensitive mutations. A suspension of ts-4 mutant virus was exposed at room temperature to 1% ethylmethane sulfonate (EMS) for 100 minutes which resulted in a 100-1000 fold decrease in virus titer. This virus was then grown in AGMK bottles and then cloned by terminal dilution in AGMK tubes. Over 100 clones were then grown in tubes and tested for restriction of growth at a lower temperature than the 38°C shutoff temperature of the parent ts-4 virus. Such mutants were not found. Attempts to remutagenize the ts-4 virus with UV light or other chemical mutagens are now in progress.

D. Parainfluenza 3 -
The desire for a virus grown only in a tissue culture substrate which is acceptable for use in a human vaccine has diverted research activity from chemical mutagenesis. A 1962 throat swab specimen from Children's Hospital of D.C. which yielded type 3 virus in human embryo kidney (HEK) culture was used to prepare a virus suspension in HEK which titered greater than $10^5$/ml. This virus, which showed negligible initial growth in WI38 cells, has been partially "adapted" to that line and cloned three times by terminal dilution in WI38 tubes. As yet titers greater than $10^5$/ml have not been attained. Attempts continue to prepare the virus in sufficiently high titer for mutagenesis in an acceptable tissue culture line.

Significance to Biomedical Research and the Program of the Institute:

The genetic studies of the important myxoviruses, paramyxoviruses and RS virus are designed to shed light upon the viruses themselves and to develop mutants which can be used for the prevention of serious disease caused by these agents. Clearly, these areas lie within the mandate of our Institute.

The most recent genetic studies of the influenza A ts-1[E] (H3N2) recombinant confirm previous indications that the genetic lesion responsible for the attenuation of this virus is limited to one gene of the virus. We have also ascertained within the past year that this altered gene can be transferred quickly and with high frequency to another influenza A virus which is of epidemiologic importance. This bodes well for future attempts to develop a live influenza virus vaccine in the face of a pandemic threat.

New insights into the biology of RS virus have been made and these should help us in our genetic manipulations and characterization of RS ts mutants. A reasonable plan has been developed for the introduction of an additional ts lesion into the ts-1 mutant and criteria for the recognition of this additional lesion in the presence of the existing lesion (or lesions) have been formulated. This approach now appears feasible.

Proposed Course of Project:

The ts-1[E], ts-1[A], ts-1[H], ts-1[C] and ts-304 mutants of influenza A (H3N2) virus will be recombined with the London (H3N2) 1972 virus and the Marton (HON1) 1943 virus. The recombinants which emerge from each mutant X wild type cross will be tested for their capacity to recombine with each other. The results of these tests should prove helpful in determining for each mutant whether ts mutation has occurred in more than one cistron. If two recombinants derived from the same mutant should recombine with each other, this would constitute evidence that the original mutant contained lesions in two or more genes and that these affected genes were segregated during recombination into different recombinants. On the other hand, failure of recombinants derived from a mutant to recombine with each other would constitute strong evidence that the original mutant had ts lesions in only one gene. The number of genes affected in each mutant represents prime information which must be obtained in order to plan for future updating of live influenza virus vaccine via recombination. Rapid

production of a new attenuated strain of virus via recombination would be facilitated by using a donor ts mutant which contains only one affected gene; otherwise it would be necessary to sift through a large number of ts recombinants in order to recover a recombinant which had received 2 ts genes by random reassortment.

The nature of the neuraminidase antigen present on the various influenza virus recombinants will be identified. In this manner it should be possible to determine if any of the ts lesions are located in that portion of the genome which codes for the neuraminidase.

Ts mutants of influenza B virus will be produced and these mutants will be manipulated in the same manner which proved to be successful with influenza A virus. Ts mutants or recombinants of influenza B virus will be investigated genetically and will also be evaluated in hamsters and subsequently in man for evidence of attenuation.

Study of the ts mutants of the parainfluenza viruses will continue in an effort to determine their potential for use in a live virus vaccine. Evaluation of the mutants for degree of defectiveness and attenuation will be conducted first in experimental animals and then in young adult men and subsequently in young children.

The ts-1 mutant of RS virus will be remutagenized by exposure to EMS, a potent chemical mutagen, in an effort to introduce additional ts lesions into the virus. This will be done in an attempt to obtain a more stable mutant, i.e., one which will not revert to wild type or exhibit pseudo-wild type properties as a result of the development of a suppressor (or correction) mutation.

Honors and Awards: None

Publications:

Kalica, A.R., Wright, P.F., Hetrick, F.M., and Chanock, R.M.: Electron microscopic studies of respiratory syncytial temperature sensitive mutants. Arch. Gesamte Virusforsch. In press.

Wright, P.F., Gharpure, M.A., Hodes, D.S., and Chanock, R.M.: Genetic studies of tespiratory syncytial virus temperature sensitive mutants. Arch. Gesamte Virusforsch. In press.

Serial No. NIAID 70B
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Classification and Characterization of Rhinovirus Serotypes of Human Origin

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: 7 Collaborating U. S. Laboratories and a WHO International Reference Center (Common Cold Research Unit, Salisbury, Wilts, England, and Clinical Research Centre, Harrow, Middlesex, England)

Contract Support:

| Contract Number | Project Officer (NIAID) | Contractor | Contractor's Proj. Dir. | Annual Funding Level |
|---|---|---|---|---|
| NIH-72-C-127CC | Dr. A.Z. Kapikian | Micro. Assoc. | Mr. M. Vincent | $6,555 |

| Man Years: | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 18/12 | | | 18/12 |
| Professional: | 1/12 | | | 1/12 |
| Other: | 17/12 | | | 17/12 |

Project Description:

Objectives: (1) To classify the numerous rhinovirus serotypes in order to develop a suitable nomenclature system; (2) to characterize rhinovirus serotypes antigenically; (3) to elucidate the interrelationships between different rhinovirus serotypes.

Methods Employed and Major Findings:

Rhinovirus Collaborative Program - The first and second phases of this collaborative program which was designed to characterize and enumerate the number of distinct rhinovirus serotypes and which was described in detail in previous annual reports instituted a new numbering system for rhinoviruses based upon extensive cross-testing of a large number of strains. The first phase of this numbering system enumerated 55 distinct serotypes from 1-55 with one subtype. The second phase added 34 additional distinct serotypes thereby extending the numbering system to include rhinoviruses 56 through 89.

A third phase of the program includes certain second phase viruses which demonstrated one-way or unresolved relationships, and prototype candidate viruses distinct from previously numbered serotypes. We submitted three viruses--607, 1169 and 604 described below--to the third phase of the program as prototype candidate viruses distinct from previously numbered serotypes. Information concerning the third phase of the program from the rhinovirus reference laboratory of Dr. V. V. Hamparian at Ohio State University (reference laboratory under contract to NIAID) indicates the following: a total of 25 prototype candidate viruses have been submitted to the third phase of the program; reciprocal neutralization tests have indicated that 22 of the 25 are distinct thus increasing the number of rhinovirus serotypes by at least 22; NIH strain 607 was found to be identical to a strain submitted previously by Dr. J. M. Gwaltney, Jr.; and NIH strains JM-1, 100301, which are holdovers from the second phase, and 604 and 1169 will be included as prototype strains in the third phase. In addition, Dr. Hamparian has received over 100 rhinovirus strains which have not been neutralized by at least 20 antibody units of antisera to the numbered 90 serotypes but which are not included in the third phase since these strains have not yet been characterized according to some or all of the criteria established by the collaborating laboratories (i.e., virus submitted must be triply "purified"; RNA content, size, ether or chloroform stability, and acid lability must be established; and homologous antisera from "purified" antigen must be submitted). However, these untypable viruses will be tested against phase 3 reagents by Dr. Hamparian's laboratory in order to assist in estimating the number of as yet untyped candidate viruses.

Study of Rhinoviruses Recovered from Epidemiologic Studies - 192 acid labile, chloroform stable viruses were recovered from patients enrolled in the NIH, LID Common Cold Study. These agents were classified as presumptive rhinoviruses on the basis of tissue culture characteristics, and acid lability and chloroform stability. With the completion of the second phase of the collaborative program and the availability of small amounts of antisera for all numbered serotypes, attempts were made to type some of these rhinovirus isolates. Such a study would not only provide valuable information on the approximate number of uncharacterized serotypes, but also yield epidemiologic information on the prevalence of different serotypes in this community. Thus far, 69 acid labile, chloroform resistant viruses have been tested in neutralization tests with antisera for the 90 numbered rhinoviruses (i.e., 1-89 plus the subtype); 54 were typed as follows: 8 as Rhinovirus [R] 2; 3 each as R7, R14, R29 and R37; 2 each as R3, R10, R41, R48, R57, and R75; and one each as R1A, R1B, R11, R16, R17, R19, R21, R22, R30, R33, R36, R43, R45, R47, R53, R54, R60, R62, R65, R73, R76, and R78. Fifteen could not be typed with the 90 available rhinovirus antisera and these are described in the two following sections of this report. Acid labile viruses obtained from studies at Junior Village are described in Project 66A. (Data in this ¶ complete through 3/31/73.)

Characterization of Three Newly Recognized Rhinovirus Serotypes of Human Origin - Three virus strains, 607, 1169 and 604, were recovered from three volunteers in the NIH, LID Common Cold Study. Strain 607 was isolated in HDCS WI26 and HDCS WI38 cultures and MA177 cell cultures from a washing

(see Project 72A) obtained on 9/17/65 from a 28 year old male; strain 1169 was recovered in semi-continuous human embryonic intestine (HEI) MA177, and semi-continuous HEI MA349 cell cultures, from a washing obtained on 9/25/70 from a 56 year old female, and strain 604 was isolated in HDCS WI38 cultures from a washing obtained on 9/16/65 from a 21 year old female. MA349 is an additional human embryonic intestine cell culture available from the laboratory of Mr. M. Vincent of Microbiological Associates, Inc. Since all 3 virus strains induced a "rhinovirus-like" CPE, were acid labile and chloroform resistant, they were tested in neutralization tests with sera containing at least 20 antibody units of each of the 90 numbered rhinoviruses and were found to be distinct in these "one-way" neutralization tests. Strains 607, 1169 and 604 were subsequently "purified" by 3 limiting dilution passages; guinea pig hyperimmune antisera were produced utilizing the "purified" virus as immunizing antigen. Strains 607, 1169 and 604 were distinct from each other by reciprocal neutralization tests. In addition, 3-320 $TCD_{50}$ of "purified" 607, 1169, and 604 viruses were not neutralized by at least 20 antibody units of each of the 90 numbered rhinoviruses; 32-320 $TCD_{50}$ of each of the 90 numbered rhinoviruses were not neutralized by 1:20 dilutions of hyperimmune guinea pig antisera prepared for each of the 3 strains. Such reciprocal neutralization tests established the serologic distinctness of these 3 rhinoviruses from the previously described 90 strains. "Purified" antigens of the 3 strains were further characterized as follows: (1) each strain measured approximately 25-30 nm as determined by "immune electron microscopy"--a technique utilized so that the virus particles would be in the form of aggregates rather than indistinctive individual particles; (2) each strain had an RNA core as determined by the 5IUDR test; (3) each strain was acid labile and chloroform stable; (4) although each strain was relatively heat stable at 50°C for 1 hour (decreasing in titer 1.0 - 1.5 $\log_{10}$ per 0.2 ml), they were further stabilized under these conditions in the presence of magnesium chloride. Paired sera from the two patients from whom strains 1169 and 604 were recovered demonstrated significant neutralizing antibody rises against the homologous virus strain, whereas such sera from the patient from whom strain 607 was recovered did not demonstrate such a rise. All 3 strains were submitted to the third phase of the NIAID-WHO Collaborative Rhinovirus Program, and 2 will be included as prototype strains as noted above. (Information from Dr. V.V. Hamparian.)

Continuing Characterization of Possible New Rhinovirus Serotypes - Twelve additional strains--715, 734, 743, 775, 779, 786, 812, 874, 926, 930, 1010, 1288--recovered from washings obtained from volunteers in the NIH, LID Common Cold Study have not been neutralized by antisera to the 90 numbered rhinoviruses. All but two (812, 926) were recovered on initial passage in monolayer cell cultures; strain 812 was not recoverable in monolayer cell cultures on initial passage and therefore was passaged 3 times in HET OC and such OC harvests produced CPE in both MA177 and HDCS WI38 cultures (see Project 72A); strain 926 produced a CPE after a blind passage in HEK cell cultures. All 12 have been shown to induce a rhinovirus-like CPE, were acid labile and chloroform stable, and were not neutralized by antisera containing at least 20 antibody units to the 90 numbered rhinoviruses, and NIH strains 604, 607 and 1169. Only one of the above (812) has been "purified" by triple terminal

dilution. Ten of the 12 have been submitted to the reference laboratory for testing against the phase 3 candidate prototype strains in order to assist in estimating the number of yet outstanding candidate viruses. (Strain 626 described in last year's annual report as a potential candidate prototype rhinovirus has been typed as R2.)

Significance to Biomedical Research and the Program of the Institute:

Rhinoviruses are the most important known group of viruses to be associated with acute upper respiratory tract illnesses in adults. However, the rapidly increasing number of serotypes and the absence of an acceptable numbering system made interpretation of epidemiological data difficult and also prevented satisfactory epidemiological investigations of rhinovirus infections. The numbering system will permit such studies to be undertaken satisfactorily and these studies will be important in further evaluating the need for rhinovirus vaccines.

Proposed Course of Project:

The third phase of the collaborative program, which should be completed shortly, will include more viruses than expected. A fourth phase of this program may prove to be necessary depending on the results of the "one-way" neutralization testing of the incompletely characterized rhinoviruses against the phase 3 prototype candidates. In addition, studies on the different antigenic components of individual rhinovirus serotypes will be carried out using the technique of "immune electron microscopy" described in detail in a previous annual report. For this purpose R1A antiserum has been prepared in guinea pigs utilizing both heat inactivated and uninactivated R1A antigen; in addition, the effects on viral aggregation by different immunoglobulin groups, such as IgA, will be investigated.

Honors and Awards: None

Publications:

Kapikian, A.Z: Chapter on Rhinoviruses. Strains of Human Viruses, edited by Majer and Plotkin (Karger, Basel 1972), pp. 193-228.

Serial No. NIAID 70C
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Mycoplasmas

Previous Serial No.: Same

Principal Investigators: Dr. H. Greenberg
Dr. Robert Chanock

Other Investigators: Dr. Benjamin Prescott (LM)

Cooperating Units: None

Contract Support: None

| Man Years: | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 44/12 | | 12/12 | 56/12 |
| Professional: | 20/12 | | 12/12 | 32/12 |
| Other: | 24/12 | | | 24/12 |

Project Description:

Objectives: (1) To study the biological and biophysical properties of mycoplasmas, with particular emphasis on Mycoplasma pneumoniae, the etiologic agent of cold agglutinin positive pneumonia, and to determine the relationship of those properties of virulence; (2) To employ newly developed, highly sensitive assays for measurement of antibody to M. pneumoniae in the study of local immunity and epidemiology of infection; (3) To determine whether local antibodies develop in nasal secretions after infection; (4) To further characterize the glycolipid antigens of M. pneumoniae and to evaluate the occurrence of shared antigens in nature; (5) To study the infectivity stability and protective capacity of ts mutants of M. pneumoniae (strain PI 1428) in the hamster animal model and in man; (6) To study the application of formaldehyde killed vaccine administered locally in hamsters; (7) To evaluate possible functions of secretory antibody directed against M. pneumoniae with specific emphasis on cystadsorption as a principal factor in virulence.

Methods Employed:

(1) Mycoplasmas were grown on agar plates or in broth suspensions. M. pneumoniae was also grown in Povitsky bottles under conditions in which the colonies became attached to the glass surface.

(2) The complement mediated killing reaction first described by Gale and Kenny for M. pneumoniae was made more sensitive by incubating organisms in the presence of serum for 60 minutes at 4°C prior to the addition of fresh guinea pig serum which served as the source of complement. In the modified killing or mycoplasmacidal (MCT) test the decrease in viability of M. pneumoniae produced by various concentrations of antiserum was determined in the presence of a constant amount of fresh guinea pig serum, and the highest dilution of test serum which produced a 90% killing effect was estimated after logit transformation using a computer program.

(3) A radioimmunoprecipitation method which employed small quantities of reagents (0.025 ml) and microtiter equipment was developed. The organisms were grown in 2 liter Povitsky bottles in the presence of $C^{14}$-oleic acid and $C^{14}$-palmitic acid. The cell sheet adhering to the glass surface was washed 3-4 times after removal of the medium. These organisms were filtered through a 450 nm millipore filter and diluted to an activity of 500-1000 cpm per 0.025 ml. Radiolabelled antigen was incubated with different serum concentrations for 60 minutes at 37°C and then overnight at 4°C. The antigen-antibody complexes were precipitated by anti-human globulin. After centrifugation of the precipitates of 1000 rpm for 10 minutes, the radioactivity remaining in the supernatant was determined. From this determination the highest serum dilution which gave a 33% binding of antigen was calculated and considered to be the titer.

(4) Syrian hamsters and guinea pigs were inoculated intranasally with either temperature sensitive (ts) or wild type M. pneumoniae. Response of the animals to infection was studied by titration of the number of organisms which had grown in the lungs and nasal turbinates, appearance of MI, CF and mycoplasmacidal antibody in the serum, and lung histopathology. Six weeks and 6 months after initial infection, the animals were challenged intranasally with a virulent wild type M. pneumoniae strain and the response to challenge studied as above.

(5) Syrian hamsters were inoculated intranasally with 0.2 ml of formaldehyde inactivated M. pneumoniae vaccine. These animals were subsequently challenged with wild type organisms and their response to wild type challenge assayed by titration of the quantity of organisms which had grown in the lungs, by study of lung histology and by measurement of antibody response.

(6) Various vegetable and bacterial mixed lipids were isolated by combined chloroform-methanol extraction, sephadex G25 chromotography and DEAE chromotography. Selected vegetable glycolipids were separated from the mixed lipids and purified by additional silicic acid chromotography. The cross reactivity of these glycolipids to M. pneumoniae glycolipids was evaluated by CF and MCT. Further, certain of the glycolipids were reaggregated with A. laidlawii membrane protein subunits into an artificial membrane and then inoculated into rabbits which were studied for their serologic response.

Major Findings:

Mycoplasma pneumoniae

(A) Secretory antibody response - As shown by the studies of Fernald, Clyde and Collier during experimental infection of the hamster with M. pneumoniae the site of localization of the organism appears to be superficial, involving only the epithelial layer of the respiratory passages. Similarly the organism remains localized to the epithelial surface following infection of organ cultures of human fetal trachea. The superficial nature of M. pneumoniae infection in the lower respiratory tract suggests that local immune mechanisms (secretory IgA and/or cell mediated immunity) in the respiratory tract should be of greater importance than serum antibody in protection from disease.

Recently, the development of a highly sensitive radioimmunoprecipitation (RIP) assay made it possible to detect the development of specific IgA antibody for M. pneumoniae in the nasal secretions of a majority of volunteers infected with this organism. Previous attempts to detect antibody in local secretions had failed but this failure may have resulted from an insensitivity of the serologic methods used. Nasal wash and sputum specimens from volunteers previously infected with the PI 1428 strain of M. pneumoniae were studied for evidence of a local secretory antibody response. Of 67 volunteers investigated, 29 (43%) had detectable antibody in acute respiratory tract secretions. This antibody was primarily IgA but in a small number of instances IgG was also found. Following challenge with wild type organisms 42% of the men developed a 3-fold or greater rise in nasal secretion antibody and 73% developed a 3-fold or greater rise in sputum antibody. In all cases antibody rises were in the IgA class. We used this radioimmunoassay to measure the level of IgA M. pneumoniae antibody in the pre-infection nasal washings collected from 45 volunteers who were challenged with wild type M. pneumoniae by Dr. Robert Couch and colleagues. For the analysis the volunteers were divided into approximately equal groups of men with low serum MI (metabolism-inhibition) antibody (1:8 or <) and high serum MI antibody (>1:8). These groups were then subdivided into sub-groups of men with low (<1:3) and high (>1:3) nasal secretion IgA antibody. Men with low levels of RIP nasal antibody (<1:3) developed M. pneumoniae disease with higher frequency following experimental challenge than did men with a higher titer (>1:3), irrespective of serum titer. The correlation of nasal antibody with resistance was significant for the group of men with a serum titer of 1:8 or > ($p=0.04$ Fischer exact test). In contrast a correlation of serum antibody with resistance was not evident. These findings suggest that local IgA antibody as measured by the RIP test was related to resistance to M. pneumoniae disease and was more important than serum antibody.

(B) ts Mutants - Last year we described the production and characterization of a series of temperature sensitive (ts) mutants of M. pneumoniae. Mutants were recovered which were restricted in growth in vitro at 37° or 38°C. At these temperatures the wild type organism grew without restriction. Missense mutants of the ts class were sought with the expectation that their temperature

sensitive defects would restrict growth in vivo at the temperature of the lower respiratory tract (37°C), whereas replication should not be impaired in the cooler environment of the upper respiratory passages (32°-34°C).

In vivo genetic stability is one of the important requirements for a mutant that is to be used in a live vaccine. Ts mutants of M. pneumoniae previously thought to be stable in vivo in the hamster had been tested after the lung tissue was frozen at -70°C and stored for one to three days before titrations were performed. When the lung tissue was tested fresh, without prior freezing, most of the previously described mutants were found to have a readily detectable frequency of reversion to wild type. This observation prompted us to search for other ts-mutants which were genetically stable in vivo.

Four genetically stable temperature sensitive mutants of M. pneumoniae were produced by exposing the organism in 100 µg/ml of N-methyl-N'-nitro-N-nitrosoguanidine (NTG). These mutants (ts H9, ts H10, ts H28, and ts H43) and a previously described mutant (ts 640) were temperature sensitive with respect to growth of colonies on agar medium. The gradient of temperature sensitivity was ts H28> ts H43> ts 640> ts H9 and ts H10. In addition, the ts H9 and ts H10 mutants exhibited a decreased capacity to adsorb to receptors on guinea pig red cells.

The ts 640, ts H9, and ts H10 mutants produced an extensive pulmonary infection in the hamster and this infection was not associated with any pathological changes. These mutants proved to be stable genetically in vivo in that revertants were not recovered from infected hamsters. Furthermore, infection with the ts 640, ts H9 or ts H10 mutant induced significant resistance to a subsequent challenge with virulent wild type M. pneumoniae.

(C) Protective effect of inactivated M. pneumoniae vaccine applied topically - Because of the finding that secretory antibody may play a critical role in immunity a preliminary examination of the effect of formaldehyde killed vaccine instilled intranasally was made. Hamsters were given 3 separate doses of 0.2 ml vaccine (lot #OSUIB) intranasally or subcutaneously over a 2 week period. Wild type infection and broth control groups were also included. Two weeks following the last administration of vaccine the hamsters were challenged with wild type organisms and were sacrificed 10 days later. There was a significant decrease in pulmonary lesions in the intranasally vaccinated animals although little if any effect on growth of the organism in the lungs was seen. The protective effect of IN vaccine as regards prevention of pulmonary lesions was as great as that produced by prior infection with wild type organisms. Because of these promising findings, a preliminary human volunteer trial is planned.

(D) Glycolipid antigenic determinants shared by M. pneumoniae, vegetables, and certain bacteria - This study was prompted by the observation that an unexpectedly large proportion of infants and young children possessed antibody to Mycoplasma pneumoniae in their serum. Since early childhood represents a time of life when the organism is not commonly associated with lower respiratory tract disease, the origin of antibody possessed by infants and young children was not immediately apparent. Such antibody could be the result of clinically

inapparent infection which occurred with high frequency. There is some evidence from epidemiologic studies performed at Children's Hospital of D.C. that such silent infection may occur with appreciable frequency during the pre-school period. On the other hand, cross-reactive or shared antigenic determinants might be responsible for some of the childhood serum antibody which reacts with M. pneumoniae. During the past year we have focused on the latter possibility and have investigated the capacity of antigenic determinants from other sources to stimulate antibody reactive with M. pneumoniae.

Within the past few years it has been established that the major serological reactivity of M. pneumoniae resides in a series of glycolipids which are located on the outer membrane of the organism. These glycolipids are responsible for most of the complement fixing activity of the organism and appear to be the critical sites which are attacked by complement dependent mycoplasmacidal antibody. Further, the majority of antibodies which bind to M. pneumoniae in the radioimmunoprecipitation assay appear to be directed against these glycolipids. Previously, Plackett and co-workers had shown that diagalactosyldiglyceride from spinach and diglucosyldiglyceride from streptococcus MG reacted with M. pneumoniae antiserum by complement fixation. In view of these observations the search for cross-reactive determinants was initially directed towards lipid and specifically, glycolipid materials.

In the initial screening tests chloroform-methanol (7:3) eluates containing mixed lipids from 53 vegetables were evaluated for their capacity to fix complement with antibodies to M. pneumoniae or its glycolipids. Forty-three of the vegetables were found to have antigenic activity with the M. pneumoniae immune sera used. Each positive vegetable reacted with the rabbit anti-M. pneumoniae glycolipid serum. Eighteen gram positive bacterial or L forms were also studied and 3 fixed complement with the M. pneumoniae antisera. The positive reactors included Staphlococcus aureus, Staphlococcus aureus L form and Streptococcus hemolyticus group A, type 1 L form.

Two of the vegetables which exhibited a high level of antigen activity were examined in greater detail. Purified glycolipids from carrots and parsnips as well as artificial membrane reaggregates containing these glycolipids exhibited a high degree of complement fixing activity. The serologically reactive constituents of vegetables and certain gram positive bacteria appeared to be predominantly glycolipid in nature since they reacted to high titer with antibodies directed specifically against the glycolipids of M. pneumoniae. Further support for this view came from the finding that the purified glycolipids from carrots and parsnips also reacted strongly with M. pneumoniae glycolipid antibodies. Additional evidence that the carrot and parsnip glycolipids were closely related to M. pneumoniae glycolipids was obtained in a series of blocking experiments. It had previously been shown that human mycoplasmacidal antibody was directed almost exclusively against the glycolipid determinants of M. pneumoniae. Further, specific M. pneumoniae glycolipid antibody was observed to be highly effective in complement mediated lysis of the organism. In the present study carrot and parsnip glycolipids incorporated in A. laidlawii membrane reaggregates were found to be efficient in blocking mycoplasmacidal antibody contained in human convalescent serum and in rabbit

antiserum prepared against the glycolipids of M. pneumoniae. The nature of the M. pneumoniae antibody found in the serum of young children was investigated by another type of blocking test. In this instance antibody was measured by the highly sensitive radioimmunoprecipitation (RIP) technique. RIP antibody reactive with M. pneumoniae antigen could be clocked with the mixed lipid extract (chloroform-methanol 7:3 eluate) from Streptococcus group A, type 1 L form or Staphlococcus aureus. These findings further support the view that certain bacteria and M. pneumoniae share similar lipid antigen.

The preceding experiments indicated that vegetables contained lipid or glycolipid antigens which reacted with antibodies stimulated by M. pneumoniae. It was of interest to determine if the reverse were true, namely could vegetable glycolipids stimulate M. pneumoniae antibodies? Carrot and parsnip glycolipids, either in a suspension of lecithin or incorporated into an artificial A. laidlawii membrane reaggregate, stimulated antibodies to M. pneumoniae which could be detected by CF, metabolism-inhibition and mycoplasmacidal assay. As expected glycolipid membrane reaggregates were more immunogenic than the glycolipid suspension alone.

M. pneumoniae glycolipids are comprised of a mixture of galactose, glucose and galactose-glucose compounds. Kenny has shown that the di-galactose and tri-galactose glycolipids of M. pneumoniae have different immunological specificities. In the present study it was not determined if the active glycolipid(s) of carrots or parsnips was chemically identical to one or more of the M. pneumoniae glycolipids. Although the chemical configuration of the carrot and parsnip glycolipids awaits further analysis, it is reasonable to predict at this time that the active glycolipids are probably similar in structure to their M. pneumoniae counterparts based upon immunological reactivity, blocking of mycoplasmacidal antibody activity and stimulation of antibodies which react with M. pneumoniae glycolipids.

Significance to Biomedical Research and the Program of the Institute:

The development and characterization of ts mutants of M. pneumoniae appears to be one of the most promising approaches to prevention of disease caused by this organism. Current findings in this area offer some hope that the ts mutants will prove useful in the control of M. pneumoniae illness.

Of particular significance in our studies was the observation that vegetable glycolipids, either in a suspension of auxiliary lipid such as lecithin or as part of an aritificial membrane reaggregate, can stimulate antibodies which react with M. pneumoniae glycolipids and which kill the organism. These findings suggest that glycolipids from vegetables and/or bacteria could be responsible for some of the antibody in human serum which reacts with M. pneumoniae or its glycolipids. However, whether this occurs in nature remains to be established. The immunogenicity of vegetable glycolipids has one potential practical application. These glycolipids may prove useful for prevention of disease in man caused by M. pneumoniae. For example, reaggregated carrot or parsnip glycolipids or glycolipids in a suspension of auxiliary

lipid might be effective in stimulating local defense mechanisms in the respiratory tract following topical application. This possibility is currently under investigation.

Proposed Course of Project:

We plan to continue our investigation of the secretory antibody response in man to M. pneumoniae infection. Attempts will be made to elucidate the chemical nature of the cross reactive vegetable and bacterial glycolipids. Studies with the ts mutants in hamsters and in man will continue and an attempt will be made to evaluate the importance of cytabsorption to virulence. In this regard the potential role of M. pneumoniae IgA antibody in preventing cytabsorption will be studied. Finally, the feasibility of using inactivated M. pneumoniae vaccine for prevention of M. pneumoniae disease will be extended to trials in man.

Honors and Awards: Invited participant at NICHD-NIAID Conference on New Approaches for Inducing Natural Immunity to Pyogenic Organisms, Winter Park, Florida, March 21-23, 1973. (Dr. Greenberg)

Publications:

Brunner, H., James, W.D., Horswood, R.L., and Chanock, R.M.: Measurement of Mycoplasma pneumoniae mycoplasmacidal antibody in human serum. J. Immunol. 108: 1491-1498, 1972.

Brunner, H., James, W.D., Horswood, R.L., and Chanock, R.M.: Experimental Mycoplasma pneumoniae infection of young guinea pigs. J. Infect. Dis. 127: 315-318, 1973.

Brunner, H., Kalica, A.R., James, W.D., Horswood, R.L., and Chanock, R.M.: Ultrastructural lesions in Mycoplasma pneumoniae membranes produced by antibody and complement. Infect. Immun. 7: 259-264, 1973.

Brunner, H., Greenberg, H., James, W.D., Horswood, R.L., and Chanock, R.M.: Decreased virulence and protective effect of genetically stable temperature sensitive mutants of Mycoplasma pneumoniae. Ann. N.Y. Acad. Sci., In press.

Brunner, H., and Chanock, R.M.: A radioimmunoprecipitation test for detection of Mycoplasma pneumoniae antibody. Proc. Soc. Exp. Biol. Med. In press.

Brunner, H., Horswood, R.L. and Chanock, R.M.: More sensitive methods for detection of antibody to Mycoplasma pneumoniae. J. Infect. Dis. 127, suppl. S52-S55, March, 1973.

Greenberg, H., Prescott, B., Brunner, H., James, W., and Chanock, R.M.: Sharing of glycolipid anitgenic determinants by Mycoplasma pneumoniae. Proceedings for the Workshop on New Approaches for Inducing Natural Immunity to Pyogenic Organisms. In press.

Serial No. NIAID 70D
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Laboratory and Epidemiologic Studies of Viral Hepatitis Agents

Previous Serial Number: Same

Principal Investigator: Dr. Robert H. Purcell

Other Investigators: Dr. Stephen M. Feinstone
Dr. Yasuo Moritsugu
Dr. Jonathan Gold

Cooperating Units: Dr. Andrew Morrow (Surgery Branch, NHI, NIH)
Dr. Paul Holland, Dr. Harvey Alter and Dr. Paul Schmidt (Blood Bank, NIH)
Dr. John L. Gerin (MAN Laboratory)
Dr. William London (NINDS)
Dr. James Maynard (CDC)
Dr. C.J. Peters (MARU)
Dr. W. Reeves (MARU)
Dr. John Fox (Univ. Wash. Seattle)
Dr. Thomas Chalmers (CC, NIH)
Dr. Arthur Levin (WHO)
Dr. Lewellys Barker (FDA)
Dr. Leon Rosen (NIAID, Hawaii)
Dr. Albert Kapikian (NIAID)
Dr. June Almeida (Wellcome Research Lab. England)

Contract Support:

| Contract Number | Project Off. (NIAID) | Contractor | Contractor's Proj. Dir. | Annual Funding Level |
|---|---|---|---|---|
| NOI AI 32504 | Dr. Purcell | Mel Labs. Inc. | Dr. Verna | $ 190,880 |
| Interagency Agreement | Dr. Byrne | Oak Ridge Nat. Labs. | Drs. J. Liverman N. Anderson | $ 475,000 |
| Interagency Agreement | Dr. Byrne | CDC | Dr. M. Suggs | None |
| NIH 71-2197 | Dr. Purcell Dr. Dunnick | Tulane Univ. | Dr. P. Gerone | $ 12,000 |
| NIH 73-2509 | Dr. Dunnick | Scripps Inst. | Dr. T. Edgington | $ 108,431 |
| PH 43-68-1309 Part A | Dr. Chanock | Flow Labs | Dr. Gilden | $ 233,529 |

| Man Years: | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 66/12 | | | 66/12 |
| Professional: | 36/12 | 6/12 | 10/12 | 52/12 |
| Other: | 30/12 | | 6/12 | 36/12 |

Project Description:

Objectives: (1) To collect clinical material of high potential infectivity for use in laboratory studies: (2) To develop and test in vitro procedures and animal systems for use in laboratory studies of viral hepatitis; (3) To propagate the viruses of hepatitis A and B in culture or in experimental animals; (4) To study the biochemistry, biophysics, immunology and epidemiology of hepatitis-associated antigens.

Methods Employed:

(1) Clinical materials were collected from patients undergoing major heart surgery at NIH and from various animal and human populations through collaborative studies as described below.

(2) Hepatitis B antigen (HB Ag) was purified by density banding in a cesium chloride gradient and by rate centrifugation in sucrose. Characterization was achieved with appropriate techniques, including analytical centrifugation, acrylamide gel electrophoresis and electrofocussing.

Particles believed to be the virions of hepatitis B were isolated, disrupted with detergent and their internal components analyzed biochemically and immunologically.

(3) Serum containing HB Ag was inoculated into various primate species and the animals were monitored for evidence of hepatitis for six months after inoculation.

(4) Serologic techniques, including complement fixation, agar gel diffusion, counterelectrophoresis, immunoelectrophoresis and radioimmunoassay were used for serologic studies.

Major Findings:

(1) Hepatitis B Antigen - An abnormal protein, hepatitis B antigen, has been found in the serum of a high proportion of persons with leukemia, Down's syndrome and hepatitis and was found to be specifically related to infection with the virus of serum hepatitis. Furthermore, the partially-purified antigen was associated with virus-like particles 20-25 nm in diameter. HB Ag was believed by some to be the virus of hepatitis. More recently, other morphological forms associated with HB Ag have been found, including a 43 nm complex particle (the Dane particle). This particle may be the virus of hepatitis B; much of the work of this project is being directed toward the study of the various forms of HB Ag.

(2) Serology -

A. Radioimmunoassay - Recent controlled studies suggest that most of the currently available radioimmunoassay tests for detecting HB Ag sacrifice reproducibility for sensitivity. One new technique, however, a "sandwich" type radioimmunoassay for the antigen developed by Ling and Overby, proved to be both specific and sensitive. We have modified this technique to the microtiter format for the identification of HB Ag.

This micro-solid phase radioimmunoassay offers several advantages, including conservation of reagents, adaptability to other antigen-antibody systems, ease of performance and economy. The technique is as sensitive and specific as any other technique currently available but can be performed with only 25 ul of sample, compared to 100 ul of sample necessary for techniques of comparable sensitivity. The micro-solid phase radioimmunoassay has been adapted for use with reagents of human origin as well as reagents derived from guinea pig hyperimmune serum. Furthermore, by use of appropriate reagents, it has been used for the detection of the internal component of the Dane particle.

In an attempt to find an antigen in hepatitis A patients (analogous to hepatitis B antigen in hepatitis B patients) we have prepared reagents from convalescent sera of hepatitis A patients and have examined acute phase sera from such patients by micro-solid phase radioimmunoassay. To date, we have not been able to detect such an antigen, perhaps in part because of the relatively transient and low level viremia associated with this form of viral hepatitis.

B. Passive hemagglutination - A passive hemagglutination technique for the detection of anti-HB Ag has proven to be a useful and sensitive method of detecting such antibody. This technique and the radioimmunoprecipitation technique are currently the only methods sufficiently sensitive to detect anti-HB Ag following primary exposure to the hepatitis B virus. We have recently modified the passive hemagglutination technique for the detection and identification of antibody to the various subtypes of hepatitis B antigen. Using this method we have successfully identified anti-subtype antibody in approximately 50% of sera containing PHA reactive antibody. Prior to the development of this technique, it was impossible to identify such antibody in sera following primary exposure to the virus. The technique has great potential for use in epidemiologic studies of the significance of the various subtypes of hepatitis B antigen.

(3) Epidemiology -

A. Longitudinal studies of transfused patients - Longitudinal studies of patients undergoing cardiac surgery with massive transfusion at NIH were begun in 1964. These studies, described in detail in previous annual reports, yielded important information on the frequency of icteric and anicteric hepatitis as well as the relative importance of hepatitis B virus, cytomegalovirus, and EB virus in the etiology of transfusion-associated hepatitis. Furthermore, a strong association between the use of commercially derived blood and hepatitis in recipients of that blood was documented on the basis of these studies. Modified blood banking procedures were initiated at the NIH Clinical Center Blood Bank, including pre-screening of all blood for the

presence of HB Ag and the use of blood derived solely from non-commercial sources. The implementation of these changes in blood banking procedures resulted in an almost 90% decrease in the frequency of post-transfusion hepatitis among patients undergoing corrective cardiac surgery with massive transfusion at the NIH. Residual hepatitis among blood recipients appeared to have two causes: HB Ag-contaminated blood in which the antigen was undetectable or could be detected only with the most sensitive radioimmunoassay techniques, and blood contaminated with an agent causing HB Ag-negative hepatitis with short incubation period, possibly hepatitis A. Studies of these patients who developed HB Ag-negative hepatitis are in progress in an attempt to identify the agent.

B. Antibody to HB Ag in human populations - Previous studies revealed that antibody to HB Ag (anti-HB Ag), as measured by radioimmunoprecipitation was found infrequently in infants and children but in increasing frequency in young and middle aged adults (almost 40% of middle aged adults in a low socioeconomic group population had such antibody). The frequency of anti-HB Ag diminished slightly in the elderly. Such a finding suggested that there was a high sub-clinical attack rate of HB Ag-positive hepatitis in the general population. Our finding of antibody predominantly in adults is in good agreement with other studies in which HB Ag was found much more frequently in adults than in children. It seems unlikely that such a high attack rate can be explained solely by parenteral spread of the virus; it is therefore probable that some other means of spread is operable.

Further studies to delineate the epidemiology of hepatitis B in open populations are in progress. One such study was carried out in collaboration with Dr. John Fox of Seattle. Families enrolled for several years in Dr. Fox's family studies were tested for anti-HB Ag. Families with serologic evidence of infection with hepatitis B virus were studied retrospectively in an attempt to determine the frequency of intra-family spread of the agent as well as the spectrum of clinical forms with which it is associated.

Preliminary evaluation of the results suggest that exposure to hepatitis B virus was infrequent in this population and that spread of the virus to other members of the family did not occur.

In another collaborative study, with Drs. W. Reeves and C.J. Peters of MARU, family contacts of index patients with HB Ag-negative hepatitis were followed longitudinally in an attempt to determine the frequency of apparent and inapparent infection among family members.

Serologic evidence of infection with hepatitis B virus was found to occur significantly more frequently among contacts of hepatitis B antigen-positive patients than among contacts of hepatitis B antigen-negative (presumably hepatitis A) patients. In fact, seroconversions were not observed at all among contacts of hepatitis A patients. These findings confirm the specificity of the radioimmunoprecipitation test for hepatitis B antibody and demonstrate the communicability of hepatitis B virus in situations not associated with identifiable parenteral transmission.

C. Study of the risk of hepatitis to medical personnel - In a collaborative study with the CCBB and Dr. Thomas Chalmers (CC), individuals considered at risk for infection with hepatitis (physicians, nurses, clinical chemistry and pathological laboratory personnel, etc.) and a carefully matched control population of individuals not exposed to patients were tested for HB Ag, anti-HB Ag and transaminase elevations.

Because of the low frequency (1%) of hepatitis B antigen among both populations studied, a significant difference in antigen frequency could not be detected. However, medical and paramedical personnel were found to have twice the frequency of antibody to hepatitis B antigen as the matched control group, confirming the general belief that hospital personnel are at higher risk of acquiring hepatitis B virus infection than the general population. The positive correlation between frequency of anti-HB Ag and age and the negative correlation between frequency of anti-HB Ag and socioeconomic status were confirmed in this study.

D. Seroepidemiology of HB Ag in primitive populations - In collaboration with Drs. W.J. Reeves and C.J. Peters (MARU), we have studied the relative frequency of HB Ag and anti-HB Ag among three primitive peoples of Panama: The Choco, the Guaymi, and the Cuna Indians. The age-specific frequency of HB Ag and anti-HB Ag varied from population to population. Studies are in progress to determine if these differences are related to genetic or cultural differences among the tribes or to differences in frequency of exposure to the virus.

E. Hepatitis B infection in Africa - The frequency of hepatitis B antigen among Africans is extremely high, averaging 5-10% of those populations studied to date. Cirrhosis of the liver is extremely prevalent in these populations, and the frequency of hepatic cell carcinoma is much higher among Africans than among populations in other parts of the world. It has therefore been suggested that hepatitis B virus may be a cause of cirrhosis and/or hepatic cell carcinoma. In an attempt to delineate the role of hepatitis B virus and, specifically, the role of the different subtypes of hepatitis B antigen and the host response to them, we are studying serum and liver biopsy specimens obtained from African populations that are being intensively studied by the International Agency for Research on Cancer (World Health Organization). These studies are being carried out in collaboration with Dr. Arthur Levin of WHO.

F. Seroepidemiology of HB Ag in non-human primates - Sera from over 2000 old world and new world non-human primates representing the major taxonomic classificationsof these animals, have been studied for HB Ag and anti-HB Ag. Antigen was rarely found but antibody was found in a variable proportion of such animals. In general, there was a direct relationship between the frequency of anti-HB Ag and the evolutionary status of the species tested.

On the basis of these studies, several species of non-human primates were selected for attempts to experimentally transmit hepatitis B virus to sero-negative animals. This approach has resulted in the successful transmission of hepatitis B virus to the chimpanzee, the rhesus monkey and the woolly monkey. Other species, including the pigtail macaque, bonnet macaque, baboon and owl monkey are currently being studied.

(4) Antigenic Subtypes of HB Ag - It has now been confirmed in several laboratories that 4 antigenic specificities of HB Ag exist. These specificities appear to have epidemiologic importance.

Progress in the development of sensitive subtyping techniques has been made: cross-absorbed antisera specific for the subtype specificities have been prepared and are being utilized in the development of sensitive radioimmunoassay techniques for the subtyping of HB Ag.

(5) Preparation of Reference Reagents for HB Ag Subtypes - In collaboration with Dr. John Gerin (MAN) large quantities of reference reagent quality anti-HB Ag were prepared in guinea pigs and rabbits. These reagents are suitable for the determination of the subtypes of hepatitis B antigens. They have been among the most frequently requested reagents in the RRB catalogue during the past year.

(6) Other Antigenic Specificities Associated with HB Ag - Recently Almeida demonstrated that the Dane particle, which shares antigenic specificity with HB Ag, contained a virus-like internal component antigenically distinct from HB Ag. In a collaborative study with Dr. Gerin, units of HB Ag-positive plasma rich in Dane particles were identified and Dane-particle rich concentrates prepared. These concentrates were used to develop two new serologic techniques (a complement fixation test and a radioimmunoassay) specific for the internal component of the Dane particle. These techniques are being employed to study the immune response of hepatitis B patients and to monitor progress in the purification and characterization of the internal component of the Dane particle. Evidence from several sources suggests that the internal component of the Dane particle is the nucleocapsid of the hepatitis B virus.

(7) Transmission of Hepatitis B Virus to Non-human Primates and Preliminary Biophysical and Biological Characterization - In a collaborative study with Dr. William London (NINDS) non-human primates of several species have been inoculated with HB Ag-positive plasma in an attempt to transmit hepatitis B to these animals.

Serial transmission of hepatitis B virus was successfully carried out in the rhesus monkey: the virus has been serially transmitted in this species through seven passages. A pool of infectious plasma representing 5th serial passage in the virus was prepared and tested. The pool had an infectivity titer of $10^{2.5}$ - $10^{3.0}$ and produced evidence of infection in over 90% of animals inoculated with it. This pool is currently being employed to characterize the hepatitis B virus.

Preliminary results obtained with the rhesus monkey-adapted hepatitis B virus suggest that it is considerably less stable than was previously thought. Previous reports of its stability, using human volunteers as an endpoint, were based upon experiments carried out with infectious materials in whole plasma or serum and/or pools of materials of unknown infectivity titer. Thus, the virus may have been protected by high protein concentrations, and extreme stability of the virus may have been assumed from experiments in which residual infectious virus remained despite extensive inactivation.

Preliminary experiments revealed approximately 100 infectious doses of the rhesus monkey-adapted hepatitis B virus were inactivated by heating at 100°C for 10 minutes or 80° or 60°C for 1 hour. Approximately 1000 rhesus monkey infectious doses of the virus were inactivated by chloroform, a finding in opposition to previous results obtained with human volunteers but in good agreement with the current belief that the lipid-containing Dane particle is the virus of hepatitis B. Attempts to determine the buoyant density of hepatitis B virus in cesium were unsuccessful because infectious virus could not be recovered from the gradient. The relative toxicity of cesium and sucrose (two of the most frequently used materials for determining buoyant density) for hepatitis B virus is currently being examined. Characterization experiments currently in progress include confirmation of the sensitivity of HBV to chloroform, sensitivity to inactivation with ether, stability to acid, confirmation of heat lability (60°C for an hour), heat stability at 50°C and at 37°C, stability of infectivity at 4°C for 1 year, and susceptibility to inactivation by several disinfectants.

A preliminary examination of routes of transmission has been completed: infection was successfully transmitted by the intravenous or intramuscular inoculation of approximately 100 infectious doses of the virus but not by the oral route. These findings are in good agreement with reported epidemiologic and volunteer studies of hepatitis B infection.

In addition, the immune response of rhesus monkeys to rechallenge with the virus is being studied in an attempt to develop in vitro correlates of immunity to infection. Attempts to neutralize the virus with specific antiserum in vitro are also in progress.

A strong positive correlation between detection of HB Ag in sera collected from experimentally infected rhesus monkeys and the infectivity of those serum specimens for other rhesus monkeys has been observed. These results are in good agreement with epidemiologic studies of hepatitis following transfusion of HB Ag positive or negative blood.

An analysis of results obtained during serial passages of hepatitis B virus in the rhesus monkey revealed an adaptation of the virus to this species of non-human primate. Thus, higher passage levels of the virus were associated with diminished immune response of the host when compared with earlier passage levels. To examine this phenomenon more fully, we have prepared a pass-3 pool of hepatitis B virus and will determine its infectivity titer and its ability to stimulate an immune response in rhesus monkeys.

We have successfully transmitted human hepatitis B virus to a woolly monkey and have prepared a pool of acute phase woolly monkey plasma for further studies. The infectivity of this pool is currently being examined.

Despite much progress in the study of hepatitis B virus in rhesus monkeys, chimpanzees remain the most sensitive non-human host for the virus. In addition, chimpanzees respond to infection with hepatitis B virus in a manner virtually identical to the response of man. Thus, a spectrum of responses,

including frank hepatitis, has been observed in chimpanzees experimentally infected with hepatitis B virus. Because chimpanzees suitable for such studies are rare and expensive, we have entered into a collaborative study with Dr. L. Barker (FDA) and Dr. J. Maynard (CDC) for the study of hepatitis infection in chimpanzees. We believe that such a collaborative effort will result in the most efficient use of such animals. Current studies are concerned with the establishment and characterization of pools of hepatitis B virus associated with each of the recognized subtypes of HB Ag. The infectivity titer of these pools will be determined in chimpanzees. The pools will then be used for studies of the host response to hepatitis B virus infection, including attempts to determine if the different subtypes of HB Ag represent different serotypes of virus, i.e., if infection with one type of virus will protect against other types. These studies are necessary first steps in determining the feasibility of hepatitis B virus vaccines.

As a part of these studies, and in collaboration with Dr. Thomas Edgington (Scripps Institute), specimens of liver and other tissue from non-human primates infected with the agent of hepatitis B are being studied for immunofluorescent evidence of HB Ag in the tissues. These studies include immunofluorescent staining for each of the subtypes of HB Ag, as well as attempts to identify and localize the internal component of the Dane particle. Such antigens have been detected by immunofluorescent means in several experimentally infected chimpanzees. The results of these studies are expected to increase our knowledge of the immunopathology of hepatitis B infection.

(8) Search for the Virus of Hepatitis A - Techniques combining immunological reactions with electron microscopic techniques (immune electron microscopy) have provided a sensitive means of detecting and identifying viruses that cannot be isolated in tissue culture. This technique is believed to increase the sensitivity of electron microscopy for detecting viruses by a factor of 1,000. Immune electron microscopy was recently successfully employed for the visualization of an agent etiologically associated with non-bacterial gastroenteritis of man. At present, IEM is the only means for identifying this agent.

In a collaborative study with Drs. Albert Kapikian and Leon Rosen (NIAID) we have employed IEM to identify a morphologically distinct virus-like particle in the stool of a patient with viral hepatitis. This individual was one of a number of hepatitis patients associated with an epidemic of viral hepatitis in the South Pacific. A serologic response to the virus-like particle was observed with paired sera from several patients associated with this hepatitis outbreak. Antibody to the particle has also been found in sera from an individual involved in a second epidemic of hepatitis, also in the South Pacific, and in the sera of volunteers experimentally infected with hepatitis A in the United States, but rises in antibody could not be detected in these individuals. Gamma globulin prepared in the United States has also been found to contain such antibody.

The virus-like particles have not been etiologically associated with hepatitis A but they do appear to infect man, probably via the fecal-oral route. They are morphologically distinct from all recognized human viruses. The virus-like particles are currently being characterized by biophysical and immunological means.

(9) Tissue Culture Studies of Hepatitis Agents - Recent evidence strongly implicates the Dane particle as the virus of hepatitis B. This particle occurs in human plasma at a much lower titer than do the other morphological forms associated with hepatitis B antigen. It has been postulated that infection of tissue culture cells with hepatitis B virus may be prevented by the large quantity of non-infectious hepatitis B antigen present in potentially infectious plasma. Such excess antigen may block the effective adsorption of the virus to tissue culture cells and prevent its entry into the cells. In an attempt to improve the chances of successfully infecting tissue and organ culture cells, Dr. Gerin (MAN) has prepared Dane particle-rich concentrates of hepatitis B antigen-positive plasma. These concentrates are being inoculated into various tissue and organ culture systems; evidence of successful infection is being sought by radioimmunoassay and by immunofluorescent techniques, using reagents prepared and certified by Dr. Edgington (Scripps Institute). These tissue culture studies are being carried out by Dr. Yasuo Moritsugu (WHO).

Significance to Biomedical Research and the Program of the Institute:

Hepatitis represents one of the major unsolved infectious disease problems. In addition, changing cultural conditions (the rise of the drug culture) has resulted in a rise in the relative importance of the more serious hepatitis B. Finally, the development of sensitive serologic techniques for the demonstration of prior exposure to hepatitis B has revealed that such infection is much more prevalent in the general population than was previously thought. Thus, hepatitis B is emerging as a public health problem of considerable magnitude. The present program is consistent with the institute's mission i.e., responsibility for the study of and, where practicable, the development of vaccines or other suitable methods for the control of infectious diseases. The recent rapid increase in our knowledge of hepatitis B has opened the way for improved control methods, including, possibly, active and passive immunization. The program of the hepatitis unit, LID, NIAID is directed primarily toward the realization of these goals.

Proposed Course of Project:

We plan to concentrate our studies of hepatitis B in the following areas: (a) studies of hepatitis B in non-human primates with the twin goals of characterizing the agent of hepatitis B and developing an animal model for the study of clinical aspects of hepatitis B infection, (b) improvement of serologic tests for HB Ag and other hepatitis-associated antigens and their use in seroepidemiologic studies, (c) extension of biophysical studies of HB Ag and anti-HB Ag interactions, (d) attempts to grow the agent of hepatitis B in tissue and organ culture, and (e) through the use of seroepidemiologic

and animal model studies, to better understand the parameters of acute and passive immunization against hepatitis B. In addition, we plan to extend and modify the techniques developed with HB Ag to studies of HB Ag-negative hepatitis. These studies will include the modification of serologic techniques for use with hepatitis A as well as attempts to establish a useful animal model system for the study of hepatitis A.

Honors and Awards:

Elected Fellow of the Washington Academy of Sciences, April, 1972.

Invited Participant, Ninth International Gastroenterology Congress, Paris, July, 1972.

Invited Participant, Symposium on Infectious Diseases, University of Vermont, October 20, 1972.

Recipient, USPHA Superior Service Award, July, 1972.

Invited Speaker, U.S.-Japan Viral Disease Working Conference, Sapparo, Japan, September 18-20, 1972.

Invited Participant, WHO Scientific Group on Viral Hepatitis, Geneva, September 25-30, 1972.

Invited Participant, American Association for the Study of Liver Disease, Chicago, Ill, November, 1972.

Invited Participant, American Society of Hematology Meeting, Hollywood, Florida, December 4, 1972.

Invited Participant, Ecological Investigations Program Seminar, Phoenix, Arizona, January 15-16, 1973.

Invited Participant, OPS Management Conference with the Secretary, NIH, October 27, 1972.

Publications:

Purcell, R.H., Holland, P.V., Wong, D.C., and Morris, J.A.: Viral hepatitis and hepatitis-associated antigen in the Far East. J. Infect. Dis., In press.

Lander, J.J., Holland, P.V., Alter, H.J., Chanock, R.M., and Purcell, R.H.: Antibody to hepatitis-associated antigen: Frequency and pattern of response as detected by radioimmunoprecipitation. J. Am. Med. Assoc. 220: 1079-1082, May 22, 1972.

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Alter, H.J., Holland, P.V., Purcell, R.H., Lander, J.J., and Feinstone, S.M., Morrow, A.G., and Schmidt, P.J.: Posttransfusion hepatitis after exclusion of commercial and hepatitis B antigen positive donors. Ann. Int. Med. 77: 691-699, Nov., 1972.

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Holland, P.V., Alter, H.J., Purcell, R.H., Lander, J.J., Sgouris, J.T., and Schmidt, P.J.: Hepatitis B antigen (HB Ag) and antibody (anti-HB Ag) in cold ethanol fractions of human plasma. Transfusion 12: 363-370, 1972.

Holland, P.V., Purcell, R.H., Smith, H., and Alter, H.J.: Subtyping of hepatitis-associated antigen (HB Ag); Simplified technique with counter-electrophoresis. J. Immunol. 109: 420-425, 1972.

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Reeves, W.C., Peters, C.J., Lander, J.J., Purcell, R.H., and Johnson, K.M.: Differences in the prevalance of hepatitis associated antigen and antibody among Panamanian Indian populations. J. Inf. Dis. In press.

Hill, D.A., Philip, R.N., Greaves, A.B., and Purcell, R.H.: A study of mycoplasmas recovered from patients with urethritis. Brit. J. Venereal Dis. In press.

London, W.T., Lander, J.J., and Purcell, R.H.: Serial transmission on rhesus monkeys of an agent associated with Australia antigen (hepatitis-associated antigen). J. Infect. Dis. 125: 382-389, April, 1972.

Maynard, J.E., Berquist, K.R., Krushak, D.H., and Purcell, R.H.: Experimental infection of chimpanzees with the virus of hepatitis B. Nature 237: 514-515, June 30, 1972.

Serial No. NIAID 70E
1. Infectious Diseases
2. Epidemiology
3. Bethesda,Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biological and Biophysical Studies of Rhinoviruses

Previous Serial Number: Same

Principal Investigator: Dr. John C. Perkins

Other Investigators: Dr. Robert F. Buscho
Dr. Albert Z. Kapikian

Cooperating Units: None

Project Description:

Details of this project were described in previous annual reports. This project has been discontinued.

Serial No. NIAID 72A
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: New Techniques for Recovery of Respiratory Tract Viruses

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigators: Dr. Robert M. Chanock
Mr. Anthony R. Kalica

Cooperating Units: Mr. W. Lee Cline, Serology Unit, LID, NIAID

Contract Support:

| Contract Number | Project Off. (NIAID) | Contractor | Contractor's Proj. Dir. | Annual Funding Level |
|---|---|---|---|---|
| PH 43-68-1309 (Part B) | Dr. Chanock | Flow Labs. | Dr. Gilden | $ 202,577 |
| NIH-72-C-127CC | Dr. Kapikian | Microbiological Associates | Mr. Vincent | 6,555 |

| Man Years: | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 14/12 | | | 14/12 |
| Professional: | 1/12 | | | 1/12 |
| Other: | 13/12 | | | 13/12 |

Project Description:

Objectives: (1) To recover new viruses from patients with respiratory disease; (2) to characterize such agents and study their biologic and biophysical properties; (3) to develop new techniques for the detection of respiratory tract viruses.

Methods Employed:

Specimens obtained from patients in the NIH, LID Common Cold Study have been studied intensively for the presence of viruses by both conventional and new techniques. The specimens are first examined by routine cell culture methods, and only those which fail to yield viruses are studied further in experimental culture systems--such systems include new cell cultures and human embryonic tracheal (HET) organ cultures. The use of new culture systems has been especially fruitful in the recovery of virus strains belonging to the human coronavirus group of viruses.

Sixteen of the 32 described coronavirus strains were first recovered by this laboratory employing new culture systems. However, study of the coronaviruses has been hampered by the lack of convenient and suitable methods for their propagation. Ten of the 32 coronavirus strains were originally isolated in HET organ cultures only, while the remaining 22, all of which were similar to a prototype strain designated 229E, were isolated in monolayer tissue cultures. Since the coronaviruses appear to be important etiologic agents of adult upper respiratory tract illnesses, continued intensive efforts have been made to develop new ways to detect these agents. Progress in the detection of coronaviruses is outlined in the following sections; in addition, the recovery of rhinoviruses by the use of new techniques is also presented.

Major Findings:

Detection of Coronaviruses by Immune and Conventional Electron Microscopy - The propagation of coronavirus strain 692 (which was distinct from coronaviruses 229E and OC43 by immune electron microscopy) in HET organ culture (OC) and its detection by immune electron microscopy utilizing the patient's convalescent serum as the source of specific antibody was described in detail in last year's annual report.

Attempts to detect additional viral agents, with special emphasis on coronaviruses, from NIH Common Cold Study patients' washings which did not yield cytopathic agents in conventional and experimental cell cultures were continued. Washings were obtained by instilling 0.85% NaCl into each nostril sequentially, with collection of the fluid emerging from the nostril and occasionally from the mouth also. The washing was then diluted approximately 60% with veal infusion broth containing 0.5% bovine serum albumin. Washings were carried through 3 passages in HET OC and the harvests of the third passage examined for the presence of virus particles utilizing in addition to conventional electron microscopy, the technique of immune electron microscopy, in which the patient's convalescent serum is the source of specific antibody. Immune electron microscopy was employed so that virus particles would appear in the form of "aggregates" rather than individual units thereby facilitating the visualization of low-titered preparations.

An additional coronavirus strain, 700, was detected by conventional and immune electron microscopy. A washing from volunteer 700 in the NIH, LID Common Cold Study was inoculated into cell cultures of MK, HEK, HDCS WI26 and HEP2; no cytopathic effect (or hemadsorption [HAD] in MK cell cultures) was detected following 2 passages in HEK and one passage in the remainder of these cell cultures. Later the washing was inoculated into MA177 (human embryonic intestine), HDCS WI38, BSC-1 and HEK cell cultures and no definite cytopathic effect (or HAD in BSC-1 cells with rat erythrocytes) was detected although nonspecific degeneration was observed in the MA177 and HDCS WI38 cell cultures. The washing was carried through 3 passages in HEK OC and the harvests of the third passage were examined for the presence of virus particles by both immune electron microscopy and conventional electron microscopic techniques. In the immune electron microscopy procedure 0.8 ml of the HET OC harvest was incubated overnight at 4°C with 0.2 ml of a 1:20 dilution of uninactivated convalescent serum from patient 700. The mixture was centrifuged at 17,000 rpm for 1-1/2

hrs and the supernate was discarded, and the pellet resuspended with a few drops of distilled water, stained with 3% PTA and placed on a formvar-carbon coated grid; the excess fluid was removed with the edge of a filter paper disc and the grid examined under the electron microscope at a magnification of 40,000. An additional preparation in which PBS was used instead of serum was treated similarly. Electron microscopic examination of the HET OC harvest-serum mixture revealed the presence of 2 aggregates of coronavirus-like particles with an abundance of antibody coating the particles. Examination of the HET OC harvest-PBS mixture revealed the presence of single coronavirus-like particles. The third HET OC passage harvest was also inoculated into MA177 and HDCS WI38 cell cultures and no cytopathic effect was observed. The patient's acute and convalescent sera were tested by the CF technique and no significant rise in antibody to a battery of respiratory viral and M. pneumoniae antigens was observed. Studies will be carried out by immune electron microscopy to determine the antigenic relationship, if any, of strain 700 with coronavirus strains 229E and OC43. Additional attempts to characterize this agent will be carried out.

Detection of a Probable Myxovirus by Immune and Conventional Electron Microscopy - A washing from patient 740 in the NIH, LID Common Cold Study was inoculated (following centrifugation in the Spinco for 1 hr at 10,000 rpm) into cell cultures of MK, HEK, HDCS WI26 and WI38, HEP2, MA153 (human embryonic thymus) and MA162 (human embryonic liver); no cytopathic effect (or HAD in MK cells) was detected following a blind passage in HEK cell cultures or the original passage in the remainder of these cell cultures, although nonspecific degeneration was described on the 20th day after inoculation in the HDCS WI38, MA153 and MA162 cell cultures. The washing was later inoculated into MA177 cell cultures and again into HDCS WI38 cultures without any cytopathic effect being detected. The washing was carried through 3 HET OC passages and the harvests of the third passage were examined for the presence of virus particles by immune electron microscopy and by conventional electron microscopic techniques. The immune electron microscopy procedure was similar to that described above for coronavirus strain 700. Electron microscopic examination of the HET OC harvest-serum mixture revealed the presence of aggregates of myxovirus-like particles. Examination of the HET OC harvest-PBS mixtures revealed the presence of single myxovirus-like particles. Review of the serologic (CF) studies of the patient's acute and convalescent sera revealed that the patient had a significant rise in antibody to the influenza A antigen. Additional studies are to be carried out to confirm the identity of the agent.

Recovery of Presumptive Coronavirus Strains by Tissue Culture Techniques - We have continued our efforts to recover coronaviruses by cell culture techniques. In a previous annual report we described the recovery of 9 "229E-like" viruses, 8 of which were isolated directly in MA177 cell cultures only. The MA177 cell culture system continues to be the most sensitive cell culture available for the recovery of coronaviruses. Utilizing such cultures we have been able to recover 9 additional "229E-like" viruses and 1 possible coronavirus strain from patients enrolled in the NIH, LID Common Cold Study. A brief description of these strains follows:

(1) Strain 886 was recovered from a washing obtained from a 29 year old male. Inoculation of the washing into conventional cell cultures did not yield any cytopathic agent; however, subsequent inoculation of the washing into MA177 cell cultures yielded a cytopathic agent which was found to be acid and chloroform labile; in addition it had characteristic coronavirus morphology on examination by both immune electron microscopy and conventional electron microscopic techniques. In this technique virus-serum and virus-PBS mixtures were incubated one hour at room temperature and then treated as described above except for the use of different amounts and dilutions of the reagents. It was typed as 229E virus in a neutralization test with 229E guinea pig antiserum. Acute and convalescent sera from patient 886 were tested by the CF technique with a battery of respiratory viral antigens, and M. pneumoniae, and a significant rise in CF antibody to 229E virus was detected. Acute and convalescent sera from patient 886 were tested in a neutralization test with the homologous virus and no rise was detected. Suckling mice inoculated intracerebrally and subcutaneously with this agent did not develop disease.

(2) Strain 884 was recovered from a washing obtained from a 21 year old female. Inoculation of the washing into conventional cell cultures did not yield any cytopathic agent. However, subsequent inoculation of the washing into MA177 cell cultures yielded a cytopathic agent which was found to be acid and chloroform labile. In addition it had characteristic coronavirus morphology on examination by both immune electron microscopy and conventional electron microscopic techniques. It was typed as 229E virus in a neutralization test with 229E guinea pig antiserum. Acute and convalescent sera were tested by the CF technique as above and demonstrated a significant increase in antibody to 229E virus. In additon, the acute and convalescent sera were tested in a neutralization test with the homologous virus and a significant increase in antibody was not detected. Suckling mice inoculated intracerebrally and subcutaneously with this agent did not develop disease.

(3) Strain 1133 was recovered from a washing obtained from a 51 year old male. Inoculation of the washing into conventional cell cultures did not yield a cytopathic agent on initial passage. However, after blind passage into HEK and subsequent initial passage of the washing into MA177 cell cultures a cytopathic agent was detected; the MA177 harvest was studied further and found to be acid and chloroform labile and to have typical coronavirus morphology by immune electron microscopy and by conventional electron microscopic techniques. This virus was also subsequently typed as 229E virus in a neutralization test with virus 229E guinea pig antiserum. The patient's acute and convalescent sera were tested by the CF technique as above and no increase in antibody was found. The acute and convalescent sera were tested in a neutralization test with the homologous virus and a significant increase in antibody was demonstrated.

(4) Strain 1142 was recovered from a washing obtained from a 49 year old male. Inoculation of the washing into conventional cell cultures did not yield any cytopathic agent on initial passage; however, blind passage into HEK cell cultures and initial passage of the washing into MA177 cell cultures yielded a cytopathic agent; the MA177 cell culture harvest was studied further

and was found to be acid and chloroform labile and to have typical coronavirus morphology by immune electron microscopy and by conventional electron microscopic techniques. This virus was subsequently typed as 229E virus in a neutralization test with 229E virus guinea pig antiserum. Acute and convalescent sera were tested by the CF technique as above and no significant increase in antibody was found.

(5) Strain 1152 was recovered from a washing obtained from a 50 year old male. Inoculation of the washing into conventional cell cultures did not yield a cytopathic agent on initial passage; however, blind passage into HEK cell cultures and initial passage of the washing into MA177 cell cultures yielded a cytopathic agent which was acid and chloroform labile. This virus was subsequently typed as 229E in a neutralization test with 229E virus guinea pig antiserum. The agent had characteristic coronavirus morphology on examination by both immune electron microscopy and conventional electron microscopy. Suckling mice inoculated intracerebrally and subcutaneously with this agent did not develop disease.

(6) Strain 878 was recovered from a washing obtained from a 57 year old male. Inoculation of the washing into conventional cell cultures did not yield cytopathic agent; however, inoculation of the washing into MA177 cell cultures yielded a cytopathic agent which was acid and chloroform labile. This agent was subsequently typed as 229E virus in a neutralization test with 229E virus guinea pig antiserum. The agent had characteristic coronavirus morphology on examination by immune electron microscopy; no definite particles were observed on examination by conventional electron microscopy. Acute and convalescent sera were tested by the CF technique as above and a significant increase in 229E virus antibody was found. Suckling mice inoculated intracerebrally and subcutaneously with this agent did not develop disease.

(7) Strain 859 was recovered from a washing obtained from a 30 year old male. Inoculation of the washing into conventional cell cultures did not yield a cytopathic agent on initial passage; however, blind passage in HEK cell cultures and initial passage of the washing into MA177 cell cultures yielded a cytopathic agent which was found to be acid and chloroform labile. Attempts to visualize the agent by immune electron microscopy, using the patient's convalescent serum, 229E guinea pig serum, and OC43 mouse serum have not been successful. The agent has not been neutralized by either virus 229E guinea pig or virus OC43 mouse antisera in conventional cell culture neutralization tests. Acute and convalescent sera from patient 859 were tested by the CF technique and no significant increase in antibody was found.

(8) Strain 1257 was recovered from a washing obtained from a 29 year old male. Inoculation of the washing into conventional cell cultures did not yield a cytopathic agent; however, initial passage of the washing into MA177 cell cultures yielded a cytopathic agent which was found to be acid and chloroform labile. This virus was subsequently typed as 229E virus in a neutralization test with 229E virus guinea pig antiserum. Acute and convalescent sera were tested by the CF technique and no significant rise to 229E or OC43 viruses was found; however, by neutralization test against the homologous virus, a

significant increase in antibody was found. Typical coronavirus-like particles were visualized by immune electron microscopy but no definite particles were seen without the addition of convalescent antiserum. Inoculation intracerebrally and subcutaneously of this agent in suckling mice did not produce disease.

(9) Strain 1259 was recovered from a washing obtained from a 24 year old female. Inoculation of the washing into conventional cell cultures did not yield a cytopathic agent; however, initial passage of the washing into MA177 cell cultures yielded a cytopathic agent which was found to be acid and chloroform labile. The virus was typed as 229E virus in a neutralization test with 229E virus guinea pig antiserum. Acute and convalescent sera were tested by the CF technique and a significant rise to 229E virus was demonstrated. Electron microscopic studies have not yet been done.

(10) Strain 1269 was recovered from a nasal pharyngeal washing obtained from a 54 year old female. Inoculation of this washing into conventional cell cultures did not yield a cytopathic agent; however, initial passage of the nasal washing into MA177 cell cultures yielded a cytopathic agent which was found to be acid and chloroform labile. The virus was typed as 229E in a neutralization test with 229E guinea pig antiserum. Acute and convalescent sera were tested by the CF technique and a significant rise to 229E virus was demonstrated; in a neutralization test against the homologous virus a significant increase in antibody was also demonstrated. Electron microscopic studies have not yet been done.

Utilization of HET OC for Recovery of Rhinoviruses - Washings obtained from patients enrolled in the NIH, LID Common Cold Study are routinely inoculated into MK, HEK, HEP2, MA177 cell and HDCS WI38 cultures; a blind passage is routinely performed in the HEK cell cultures. If no cytopathic agent is detected in such cultures, the nasal washing is routinely passaged 3 times in HEK OC. The harvest fromthe third organ culture passage is inoculated into the cell cultures mentioned above and if no CPE is observed, the HET OC harvest is examined under the electron microscope by both immune electron microscopy and conventional electron microscopic techniques. The four presumptive rhinoviruses outlined below were recovered in cell cultures inoculated with HET organ culture harvests:

(1) Strain 812 was recovered from a washing obtained from a 32 year old male in MA177 and HDCS WI38 cell cultures following 3 HET OC passages; if produced a "rhinovirus-like" CPE and was acid labile and chloroform stable; it was not neutralized by sera containing at least 20 antibody units to each of the 90 numbered rhinoviruses. It has been "purified" in three terminal dilution passages in HDCS WI38 cultures.

(2) Strain 674 was recovered from a washing obtained from a 21 year old female in MA177 cell cultures following 3 HET OC passages; it produced a "rhinovirus-like" CPE and was acid labile and chloroform stable. This washing had originally induced a CPE in human aorta (A-39) cell cultures but the harvest was negative on subsequent passage. This isolate has been typed as rhinovirus 78.

(3) Strain 746 was recovered from a washing obtained from a 19 year old female in MA177 cell cultures following 3 HET OC passages; it produced a "rhinovirus-like" CPE and was acid labile and chloroform stable and has been typed as rhinovirus 75.

(4) Strain 848 was recovered from a washing obtained from a 23 year old female in both HDCS WI38 and MA177 cell cultures following 3 HET OC passages; it produced a "rhinovirus-like" CPE and was acid labile and chloroform stable. This isolate has been typed as rhinovirus 76.

(5) Strain 880 was recovered from a washing obtained from a 20 year old female in MA177 cell and HDCS WI38 cultures following 3 HET OC passages; it produced a "rhinovirus-like" CPE and was acid labile and chloroform stable. This isolate was typed as rhinovirus 74.

Significance to Biomedical Research and the Program of the Institute:

The search for new agents using the techniques outlined above is of importance in identifying possible etiologic agents of respiratory disease and as an initial step toward the ultimate goals of prevention and therapy. The recovery of new agents such as those described above attests to the value of this endeavor. Further study of the biologic and biophysical nature of these viruses, and the epidemiology and pathogenesis of diseases associated with them can be expected to bring us closer to these goals.

Proposed Course of Project:

The search will continue for new agents from patients with respiratory disease using the techniques outlined in this report, and any new systems which appear to be profitable. In addition, further attempts will be made to adapt the organ culture strains to cell culture and to devise new methods for their study.

Honors and Awards:

Invited member of the World Health Organization, Food and Agriculture Organization of the United Nations Working Team on Coronaviruses in the WHO/FAO Program on Comparative Virology.

Invited Member of International Coronavirus Study Group.

Member NIAID Clinical Research Committee.

Publications:

Kapikian, A.Z., James, H.D., Jr., Kelly, S.J., and Vaughn, A.L.: Detection of Coronavirus Strain 692 by Immune Electron Microscopy. Infection and Immunity 7: 111-116, 1973.

Serial No. NIAID 72B
1. Infectious Diseases
2. Respiratory Viruses and Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Study of Respiratory Viruses and Mycoplasmas in Human Volunteers

Previous Serial Number: Same

Principal Investigators: Dr. Brian R. Murphy
Dr. Harry Greenberg
Dr. Douglas Richman
Dr. David Hodes
Dr. Robert M. Chanock

Cooperating Units: Dr. Louis Potash, Flow Laboratories
Dr. Richard Hornick and Dr. Y. Togo, and Dr. Andrew Schwartz University of Maryland School of Medicine
Dr. Robert H. Parrott and Dr. H.W. Kim, Children's Hospital D.C.
Dr. Gordon Douglas, University of Rochester

Contract Support:

| Contract Number | Project Off. NIAID | Contractor | Contractor's Proj. Dir. | Annual Funding Level |
|---|---|---|---|---|
| PH 43-68-1309 Part B | Dr. Chanock Mrs. Mattheis | Flow Labs. | Dr. Gilden | $ 202,577 |
| NOI AI 32503 | Dr. Chanock | Univ. of Md. | Dr. Hornick | $ 58,247 |
| NIH 71-2091 | Dr. Chanock | Research Foundation of Children's Hospital, D.C. | Dr. Parrott | $ 133,325 |
| NIH 72-2503 | Dr. Murphy Dr. Horton | University of Rochester | Dr. Douglas | $ 130,005 |
| Interagency Agreement | Dr. Chanock | Lorton Reformatory | | $ 19,500 |

| Man Years: | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 81/12 | | | 81/12 |
| Professional | 45/12 | | | 45/12 |
| Other: | 36/12 | | | 36/12 |

Project Description:

Objectives: (1) To evaluate temperature sensitive mutants and recombinants of influenza A virus for evidence of attenuation in adult volunteers; (2) To characterize the serum and local respiratory tract anti-neuraminidase antibody response of man to infection with wild type or attenuated influenza A virus; (3) To evaluate the infectivity and clinical response to ts mutants of M. pneumoniae.

Methods Employed:

(1) Influenza Virus - The subjects for these studies were volunteers from the District of Columbia Reformatory in Lorton, Virginia and the Maryland House of Correction, Jessup, Maryland. The influenza virus suspensions used were subjected to extensive safety tests and adventitious agents were not detected. Men were selected on the basis of general good health and absence or low level of serum neutralizing antibody titer for influenza A (H3N2). Informed consent was obtained prior to participation in the study. The men were maintained in isolation in a dormitory for 2 weeks. Volunteers were examined daily by two physicians, and cultures, respiratory secretions, and blood samples were taken when appropriate. The men were inoculated intranasally with $10^{5.0}$ $TCID_{50}$ to $10^{7.0}$ $TCID_{50}$ of virus with one-half of the inoculum administered as a coarse droplet aerosol (DeVilbiss atomizer), while the remainder was pipetted slowly into the nares. Nasopharyngeal washings for virus isolation were collected in veal infusion broth daily and inoculated into rhesus monkey kidney roller tube cultures. Cultures were examined for presence of a hemadsorbing virus every 5 days and discarded after 20 days if negative. Virus isolates were identified by the hemadsorption-inhibition test. Serum antibody and nasal antibody levels were determined by the neutralization technique.

(2) M. pneumoniae - Volunteers were selected for the lowest possible level of serum metabolism-inhibition antibody. $10^7$ CFU of the ts 640 mutant were given by the nasopharyngeal route to 11 adult volunteers. The men were housed in an isolated dormitory and were sampled every other day for presence of mycoplasma in their nasopharynx. Nasal wash, sputum and serum were collected periodically for study of immunological response to the M. pneumoniae mutant.

Major Findings:

(A) Temperature Sensitive Recombinants of Influenza A Virus - Missense ts mutants of influenza A virus were prepared with the expectation that their temperature sensitive defects would restrict virus growth in vivo at the temperature of the lower respiratory tract (37°C), whereas replication should not be impaired in the cooler environment of the upper respiratory passages (32°-34°C). Ts mutants of a 1965 influenza A (H2N2) virus were produced by growing the virus in the presence of 5 fluorouracil. The ts lesions were then transferred to a Hong Kong influenza A (H3N2) virus by genetic recombination. Four such genetically distinct influenza A (H3N2) ts recombinants were evaluated for in vitro properties in tissue culture and for in vivo properties in the hamster and in man. The 4 ts recombinants exhibited a

spectrum of temperature sensitivity; one recombinant had a shutoff temperature for plaque formation of 37°C, one shutoff at 38°C, while the remaining 2 recombinants had a shutoff temperature of 39°C. The 37°C shutoff recombinant did not infect seronegative volunteers. The 39°C shutoff recombinants infected each of the seronegative volunteers tested and appeared to be attenuated; however attenuation was not complete since each of the volunteers developed respiratory tract symptoms, in some instances associated with low grade fever. The ts recombinant with intermediate temperature sensitivity infected almost all seronegative volunteers and appeared to be satisfactorily attenuated. Thus, there appeared to be a direct relationship between degree of temperature sensitivity in vitro and level of attenuation for man. In the initial volunteer studies it was shown that the 38°C recombinant, ts-1[E] induced complete protection to challenge with a virulent wild type suspension of Hong Kong virus. Furthermore, it was found that infection with the ts-1[E] virus did not spread from infected vaccinees to close contacts.

The response of volunteers to the attenuated influenza A (H3N2) ts-1[E] recombinant virus grown in bovine kidney (BK) tissue culture was examined in greater depth during the past year. In addition, the ts-1[E] recombinant grown in avian leukosis virus free eggs was given to seronegative volunteers and their response was compared to that of men given the bovine kidney culture grown virus. The egg grown recombinant was found to be attenuated, antigenic and genetically stable and did not differ perceptably in its effect on seronegative volunteers from the BK grown virus. Thus, the important properties of the ts-1[E] recombinant appeared to be genetically determined and not influenced by the host in which the virus was grown.

In the initial evaluation of the ts-1[E] recombinant $10^6$ $TCID_{50}$ of bovine kidney culture grown virus did not cause influenzal illness or lower respiratory tract symptoms. Attenuation of the virus was further confirmed in a recent study by the finding that $10^7$ $TCID_{50}$ of egg grown virus produced a similar, benign clinical response although virus replication was extensive enough to induce an immunologic response in serum and nasal secretions which approached that stimulated by the wild type, virulent virus. In addition several volunteers infected with $10^6$ $TCID_{50}$ of ts-1[E] virus had a level of virus replication in the nasopharynx which was comparable to that seen in volunteers who were infected with wild type virus and who developed influenzal disease. The ts-1[E] infected volunteers, in contrast, developed only mild upper respiratory symptoms. These results suggest that the absence of lower respiratory tract symptoms in the ts-1[E] vaccinees reflects a restriction in virus replication in the lungs. Previously we had shown that the ts-1[E] virus grew poorly in the hamster's lungs. Additional evidence that the lower respiratory tract is not involved during ts-1[E] infection was provided by the studies of Douglas and colleagues. They investigated 7 young adults who had low or undetectable serum HI antibody for the Hong Kong virus. Each of the volunteers was infected following administration of $10^6$ $TCID_{50}$ of ts-1[E] virus; however, none of these individuals developed any alteration in pulmonary function over a 60 day period. In these studies vital capacity, pulmonary compliance, one second forced expiratory volume, maximum mid-expiratory flow rate, $CO_2$ diffusion capacity, peak expiratory flow rate, closing volume, flow volume loops, and total peripheral resistance at varying frequencies were measured.

The ts-1[E] vaccinees, as a group, shed approximately 3000-fold less virus from the nasopharynx than volunteers infected with wild type virus, indicating that the recombinant was attenuated for the upper respiratory tract as well. Possibly, this ts virus, with a shutoff temperature of 38°C, exhibits a decreased efficiency of replication in vivo over a temperature range approaching its shutoff temperature and therefore replicates less effectively at the temperature of the upper respiratory tract.

In addition to attenuation and immunogenicity two other features make this virus a suitable candidate for further evaluation as a live virus vaccine strain: genetic stability and lack of communicability. To date, virus isolates from 44 volunteers administered the ts-1[E] virus have been examined for their temperature sensitivity, and each isolate was shown to retain the ts phenotype, thus further documenting the genetic stability of this virus in man. Previously, the ts-1[E] virus was shown not to spread from infected volunteers to susceptible contacts. The inability of the ts-1[E] virus to spread is probably due, in part, to its failure to induce cough or extensive sneezing and also to its high human infectious dose$_{50}$ ($HID_{50}$) which appears to be approximately $10^5$ $TCID_{50}$/ml. The high $HID_{50}$ of the ts-1[E] virus may be of particular importance since the quantity of virus produced in the nasopharynx is relatively small.

The observations made during the past year further characterize the response of volunteers to the potential vaccine virus, ts-1[E]. The major picture that emerges from our experience is that the pattern of infection caused by the ts-1[E] virus is comparable to that found after infection with wild type virus but is of a lesser magnitude. This is an important consideration in the development and use of a live influenza virus vaccine because one would be justly reluctant to employ a mutagenized virus which manifested a pattern of infection which varied significantly from that of the wild type virus. However, this is not the case with the ts-1[E] virus.

To date, the ts-1[E] virus has been given to 10 children, 3-1/2 to 11 years of age, who had low or undetectable serum HI antibody. Each of the children became infected but none developed a significant clinical response. A total of 73 adults with low or undetectable serum HI antibody have been given the ts-1[E] virus and their response to infection has been uneventful. Included in this group were 7 adults studied by Dr. A.S. Beare at the Common Cold Research Unit, Salisbury, Great Britain. Dr. Beare concluded the ts-1[E] virus was "satisfactorily attenuated and immunogenic." In addition, 56 individuals, most of whom were elderly and most of whom had serum HI antibody, were studied for their response to the ts-1[E] recombinant. Finally, isolates from 39 adults and 5 children have been tested for evidence of reversion and no revertants were detected.

(B) Specificity of Resistance Induced by Influenza A (H3N2) ts-1[E] Virus - As described previously, volunteers who received $10^6$$TCID_{50}$ of BK grown ts-1[E] (H3N2-68) virus neither developed illness nor shed virus when challenged 35 days after vaccination with $10^{4.5}$$TCID_{50}$ of a virulent wild type virus (H3N2-68).

In order to determine if the observed resistance to wild type challenge represented immunologically specific resistance or heterologous resistance, volunteers who received $10^7 TCID_{50}$ of ts-1[E] virus (H3N2-68) were challenged intranasally seven days after influenza virus administration with a heterologous, interferon sensitive virus - parainfluenza 1. Clinical response and pattern of virus shedding of the vaccinees were compared to a control group. Both groups of volunteers were matched for susceptibility to parainfluenza 1 infection by careful selection of men with a low titer of nasal wash antibody. The majority of men in each group (10 men per group) developed upper respiratory tract illness and a similar number of men shed virus. However, the vaccinees shed 10-fold less virus than controls. These results are interpreted to mean that little, if any, heterologous resistance could be detected even at a time when one would be more likely to detect it, and that the observed protection afforded by the ts-1[E] virus (H3N2-68) to wild type challenge (H3N2-68) was mediated by immunologically specific mechanisms.

(C) Ts Mutant of M. pneumoniae - Because of the promising results observed in studies performed in hamsters with the ts-640 mutant, this organism was evaluated in eleven volunteers who lacked serum MI antibody. In hamsters the ts-640 mutant produced an extensive infection without inducing pulmonary lesions. Further, the mutant was stable genetically and induced significant resistance to wild type challenge. The ts-640 mutant, which was of intermediate temperature sensitivity, infected each of the volunteers and the majority of infections were silent. Furthermore, each of the isolates recovered from the volunteers was temperature sensitive, indicating that the ts-640 mutant was stable genetically in man. However, 2 of the volunteers developed bronchitis which indicated that the mutant possessed sufficient residual virulence to disqualify it as a candidate vaccine strain. Nonetheless, the results of the volunteer study were sufficiently encouraging to prompt us to continue our clinical evaluation of ts mutants of M. pneumoniae which are more temperature sensitive than ts-640.

Significance to Biomedical Research and the Program of the Institute:

It appears that ts mutants of influenza A virus as a class are attenuated for both experimental animals and man. One of the mutants (recombinant ts-1[E] [H3N2]) appeared to possess the balance of attenuation and immunogenicity desired of a live vaccine strain. During the past year we have enlarged our experience with the ts-1[E] recombinant of influenza A (H3N2) virus and have further documented the benign nature of the infection produced by this virus in susceptible individuals.

Observations made during the course of these studies have confirmed the genetic stability of the ts-1[E] virus in man. Initially we had shown that the ts-1[E] virus was safe for administration to healthy, susceptible, young adults. Significantly, during 1972-1973 we observed that this mutant produced a benign infection in the young and the very old, i.e., those segments of the population which are most at risk of developing serious disease during an encounter with naturally occurring strains of virus. Thus ts-1[E] (H3N2) failed to induce any symptoms or produce any mild rhinitis when given to susceptible young children or elderly individuals.

Failure of the ts-1[E] virus to produce significant involvement of the lower respiratory tract was documented in a collaborative study performed with Dr. Douglas. In this investigation various sophisticated techniques were used in an effort to detect an alteration in pulmonary function during or following ts-1[E] infection; none was found. This observation provides additional evidence for the safety of the ts-1[E] virus in man.

The experience with the ts-640 mutant of M. pneumoniae suggested that the mutant was attenuated for man, but the level of attenuation was not sufficient to consider using the mutant in a vaccine. Nevertheless, the results of the study were promising enough that we plan to test in man other mutants which exhibit a greater degree of temperature sensitivity in vitro than the ts-640 mutant. It was particularly reassuring to note that the ts-640 mutant was stable genetically although it was excreted for up to 60 days.

Proposed Course of Project:

Major emphasis will be given to evaluation of a ts-1[E] recombinant which contains the outer glycoprotein surface antigens of London/1972 influenza A virus. This recombinant, which contains the surface antigens of the contemporary influenza A virus, will be evaluated in susceptible young adults. The results of this study should provide additional information concerning the genetic control of attenuation by the ts-1[E] lesion. Previously, transfer of the ts-1[E] lesion from an H2N2 virus to an H3N2 virus resulted in attenuation of the latter. Will transfer of ts-1[E] to a 1972 antigenic variant of an H3N2 virus also result in attenuation of the recipient recombinant virus? If this occurs one can be more confident that attenuation can be produced at will by transfer of the ts-1[E] lesion into an influenza A virus of contemporary importance.

We plan to evaluate the ts-42 mutant of M. pneumoniae in susceptible volunteers. This mutant is more temperature sensitive than the ts-640 mutant studied this year. Finally, the effect of intranasal instillation of inactivated M. pneumoniae vaccine will be studied in susceptible volunteers. Particular emphasis will be given to a study of the local, respiratory tract, secretory antibody response to intranasal by administration inactivated vaccine.

Honors and Awards:

Attended Meeting of Directors of W.H.O. Respiratory Virus and Enterovirus Reference Centres, London, April, 1973. (Dr. Murphy)
Member, NIAID Advisory Committee on Influenza (Dr. Murphy).

Publications:

Murphy, B.R., Kasel, J.A., and Chanock, R.M.: Association of serum anti-neuraminidase antibody with resistance to influenza in man. New Eng. J. Med. 286: 1329-1332, 1972.

Murphy, B.R., Chalhub, E.G., Nusinoff, S.R., Chanock, R.M.: Temperature-sensitive mutants of influenza virus. II. Attenuation of ts recombinants for man. J. Infect. Dis. 126: 170-178, 1972.

Murphy, B.R., Chalhub, E.G., Nusinoff, S.R., Kasel, J.A. and Chanock, R.M.: Temperature-sensitive mutants of influenza virus. III. Further characterization of the ts-1[E] influenza A recombinant (H3N2) virus in man. J. Infect. Dis., In press.

Murphy, B.R., Baron, S., Chalhub, E.G., Uhlendorf, C.P. and Chanock, R.M.: Temperature-sensitive mutants of influenza virus. IV. Production of interferon in the nasopharynx by wild type and a temperature sensitive recombinant virus. J. Infect. Dis., In press.

Serial No. NIAID 72C
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Role of Delayed Hypersensitivity in the Immune Response to Respiratory Viral Infections

Previous Serial Number: Same

Principal Investigator: Roger E. Wetherbee, M.D.

Other Investigators: Robert F. Buscho, M.D.
Brian R. Murphy, M.D.
Elias G. Chalhub, M.D.
Anthony R. Kalica

Cooperating Units: Dr. H.W. Kim, Virus Research Laboratory
Children's Hospital of D.C.

Project Description

Details of this project were described in last year's annual report. This project has been discontinued.

Publications:

Wetherbee, R.E.: Induction of Systemic Delayed Hypersensitivity During Experimental Viral Infection of the Respiratory Tract with a Myxovirus or Paramyxovirus. Jounral of Immunology (in press).

INDEX

LABORATORY OF VIRAL DISEASES


| | | |
|---|---|---|
| | Summary Statement | 1 |
| 68 H | Immunologic and Other Studies of Animal Leukemia Viruses | 7 |
| 71 A | Studies of Parvoviruses with Emphasis on the Adenovirus-Associated Viruses (AAV) | 9 |
| 71 D | Studies of Mouse Leukemia Viruses | 12 |
| 71 E | Studies of Viral Antigens in Virus-induced Tumors | 18 |
| 71 F | Studies on Interferon and Interferon Inducers | 25 |
| 71 G | Structure and Replication of Viruses | 30 |
| 71 G-1 | Biological Ultrastructure | 35 |
| 71 H | Host Defenses against Viral Infection: Interferon and Immunity | 38 |
| 71 I | The Interaction between Latent and Slow Viruses, especially the Lactic Dehydrogenase Virus (LDV) and the Subcellular Elements of the Host Cell (Mouse Macrophages for LDV). Use of the LDV-modified Macrophages Model for the Study of Role of Macrophages in the Defense Mechanisms of the Host | 44 |
| 71 J | Oncogenic and "Slow" Viruses | 47 |

PHS-NIH
SUMMARY REPORT

# ANNUAL REPORT OF THE LABORATORY OF VIRAL DISEASES, NIAID
July 1, 1972 - June 30, 1973

Dr. Wallace P. Rowe
Chief, Laboratory of Viral Diseases

The program of LVD deals chiefly with two areas of research--the biology of viruses which can integrate into host cells, and the study of host defense mechanisms and their manipulation for control of viral diseases.

The integration of bacteriophage DNA into the host cell genome, i.e., lysogeny, has been known for many years. Subsequently, study of DNA tumor viruses, in large part carried out in LVD, showed that mammalian cells could also carry integrated DNA viral genomes. Current work in LVD has now carried this concept a major step forward, by providing powerful evidence that viral genomes can be carried as part of the normal chromosomal complement of genes in mammalian cells, specifically that the genome of murine leukemia virus is carried as a chromosomal locus in mice. The precise chromosomal location of one such genome carried by the AKR mouse has been determined, and preliminary mapping has been accomplished for a similar gene in a C3H line. In addition, we have shown that a viral genetic marker follows these chromosomal loci, and that normal mouse DNA contains sequences homologous to the genome of the virus. While the concept that genes of murine leukemia virus are present in cell DNA has been suggested by a number of workers, the studies done in LVD on genetic transmission and IUDR activation of the virus have provided the most definitive proof of this concept.

While these developments have come from studies of the relationship of viruses to cancer, their possible significance extends beyond the cancer virus area. Expression of endogenous viral genes could play a role in embryogenesis, in production of autoimmune disease, and in the etiology of certain slow virus infections. Also, integration of exogenous viral genomes may be important in the biology of certain infections of man. Two examples of the latter are illustrated by current work of LVD scientists. Dr. Takemoto has demonstrated the close relationship of the new human papovaviruses to SV40, a virus which is known to be capable of integrating into cells of many species, including human cells in tissue culture; he has also found that antibodies to these agents are found in the majority of adult humans. It may well be that many persons carry these viruses in a lysogenic state. Adeno-associated virus (AAV) type 2 has been isolated on a number of occasions from human embryos. Dr. Hoggan has now shown that these viruses can integrate into cells in tissue culture and be activated many generations later. Again, the possibility exists that the natural history of AAV's involves a lysogenic relationship with human cells.

The function of integrated viral genomes is doubtless dependent on the site of integration, the number of copies integrated in tandem, and the transcriptional control points in the region of integration. The adeno-SV40 hybrid viruses in

essence provide a model for studying these factors using viral DNA integrated into another viral DNA rather than cellular DNA. As an example, the immense difference in efficiency of yielding infectious SV40 virus seen with different SV40-yielding Ad2-SV40 hybrids has been essentially explained by the finding by Drs. Lewis and Kelly that the low efficiency yielder (LEY) contains exactly one copy of SV40 DNA, while the high efficiency yielder (HEY) contains either 1.4 or 2.2 copies as a linear piece. This means that the SV40 piece in HEY can regularly homologize with itself, forming a circular molecule which can recombine out of the adeno genome; the SV40 segment in LEY will not have a complementary region to anneal to and thus will rarely loop out to give free SV40 DNA. This concept may explain the high frequency of activation of the mouse leukemia genome in certain mouse strains, and its rarity in other strains even though both contain the whole genome.

The project on host defense mechanisms has made progress at both the theoretical and practical levels. A most exciting lead has been obtained in the study of the molecular basis of the interferon effect, that is, that cytoplasmic messenger and transfer RNA's in interferon-treated cells are longer than in control cells, most likely as a result of added poly-A sequences. If this can be firmly established, it will provide the basis for much deeper insight into the recognition and rejection of foreign RNA's by ribosomes. On the practical side, new techniques have been developed for production of high titer interferon and for its precise assay; also, purification of human interferon is proceeding reasonably well.

A significant problem (self-made) has developed in the work with nondefective adeno-SV40 hybrids. With publication of the characteristics of these viruses we feel obligated to make them available to other laboratories. We also feel obligated to ensure that they do not get into the general population, since they, in theory, could constitute a significant biohazard. While confident of our own ability to contain these agents (by such means as good laboratory technique and screening of personnel to be sure that only immune persons handle the viruses), we are not confident that laboratories less experienced in handling pathogens can handle them safely. In order to avoid using a double standard, we have suspended work with these agents and are trying to develop mechanisms (in collaboration with the Center for Disease Control and the NIH Biohazards Committee) for containing these agents--mechanisms that will be effective but not overly restrictive, and which will apply both to ourselves and outside laboratories.

Dr. Rowe received several awards, namely, a Rockefeller Public Service Award, the Gustav Stern Lectureship to honor Peyton Rous, the G. Burroughs Mider Lectureship, and the G. H. Clowes Lectureship of the American Association for Cancer Research.

Precise mapping of a gene for infectious MLV. Further studies of the two unlinked chromosomal loci carried by the AKR mouse and apparently representing integrated virus genetic material have allowed the precise location of the linkage group I gene (Akv-1) as 12 map units from the Gpi-1 locus, with a gene order of c - Gpi-1 - Akv-1. Studies of transmission of virus by another high MLV producing mouse strain, C3H/Fg, indicate the presence of at least 3 virus-

inducing loci, one of which is also on linkage group I but much closer to the albino locus than Akv-1. This suggests that the integrated virus genomes are not at allelic sites in different mouse strains. [Rowe; Bremner (Howard U.); Hartley].

Mapping of the Fv-1 locus. The Fv-1 locus, the major determinant of the growth of naturally occurring MLV strains in vivo and in vitro, has been located in linkage group VIII, very close to the Gpd-1 isozyme locus. Use of the Fv-1 locus as a marker has already shown that genes on linkage group VIII have a marked effect on expression of MLV antigens. [Rowe; Sato, Ikeda, Stockert (Sloan Kettering Inst.); Lilly (Einstein)].

Mechanism of MLV induction by halogenated pyrimidines. The activity of IdU and BdU in inducing MLV in AKR embryo cell lines was found to require incorporation of the analog into cellular DNA; cells were found to be most susceptible to induction during the S phase of the cell cycle. [Lowy, Teich].

Quantitation of MLV-specific DNA in mouse cells. Using isotopically labelled single-stranded DNA probes synthesized from MLV RNA, tested against DNA from mouse embryo cells from various sources, differences in viral DNA-cellular DNA reassociation kinetics have been found. The results obtained suggest a smaller number of viral genome copies present in embryo cells from low leukemia strains of mice as compared to high leukemia strains. [Lowy, Chattopadhyay, Teich; Levine (NCI)].

Induction and genetic transmission of B-tropic MLV. Studies of the genetic transmission of B-tropic MLV have not been feasible because of low-titer or inefficient transmission in vivo and because the treatment of embryo cultures with IdU usually results in activation of only N-tropic MLV even from strains of mice which produce B-tropic virus post-natally. Cultures of B10·D2 and B10·Br embryos, however, have occasionally yielded B-tropic virus and genetic studies with these strains, testing for segregation for B-tropic virus inducible by IdU, may now be possible. [Moll, Rowe, Hartley].

Development of cell line highly sensitive to MLV infection. A clonal derivative of a wild mouse embryo cell line has been obtained which is sensitive to both N- and B-tropic strains of MLV. Further, it displays increased sensitivity (up to 40-fold) compared to secondary embryo cultures, particularly for detecting virus in tissue extracts or virus activated by IdU treatment of cell cultures. [Hartley].

Induction of plasmacytomas by Abelson virus. Abelson virus, a lymphosarcomagenic variant of Moloney virus, has been found to induce transplantable, immunoglobulin-producing, plasmacytomas in mineral oil-treated BALB/c mice. [Sklar, Rowe; Potter (NCI)].

SV40 early genes mapped by the use of nondefective adeno-SV40 hybrid viruses. By determining the functional capacities of the different nondefective hybrid viruses and relating their DNA segments to the $R_1$ linear segments of SV40 DNA, it has been possible to construct the gene order and location in the SV40 molecule of the early antigen-inducing regions of SV40. Since these include

the antigens expressed in transformed cells, this may represent a major step toward defining the region of the SV40 genome which is most crucial for tumorigenicity [Lewis; Kelly (Johns Hopkins U.); Rowe].

Messenger RNA produced by hybrid viruses found to be hybrid. Sequential hybridization studies have shown that mRNA produced by one of the nondefectiv hybrids contains covalently linked adeno and SV40 RNA sequences. This obser- vation explains why SV40 functions when induced by the hybrids show adenoviru type resistance to interferon [Oxman (Harvard); Lewis].

$Ad2^{+}ND_{5}$ hybrid shows translational block. This hybrid, though containing the genetic information for SV40 U antigen synthesis, does not produce the antige Study of polysome-bound mRNA has shown that the virus produces a functional mRNA which cannot be translated, suggesting that it may be a frameshift mutar This could be a useful tool for studying frameshift mutagens in mammalian systems [Crumpacker (M.I.T.); Lewis].

Molecular basis found for difference between high- and low-efficiency yielde Ad-SV40 hybrids. The HEY hybrid has been found to contain 1.4 or 2.2 SV40 genomes, in tandem, inserted in the adeno DNA, while the LEY hybrid contains 1.0 SV40 genome. Thus the HEY virus readily loops out, while the LEY will not make self-annealed circles [Lewis; Kelly (Johns Hopkins U.)].

Marek's disease herpesvirus undergoes antigenic change in tissue culture. The soluble antigen of the herpesvirus of Marek's disease of chickens is no antigenic in chickens, suggesting that immunologic tolerance is present. However, with repeated tissue culture passage, the virus may convert with respect to plaque type produced. When this occurs the soluble antigen becom capable of inducing antibody response in the chicken. This antigenicity cou be involved in the poorly understood ability of similar adapted strains to immunize against naturally occurring Marek's disease. [Cook].

Long-term passage of AAV carrier cultures of Detroit-6 cells and establish- ment of infected subclonal populations. AAV-1 carrier cultures of Detroit-6 cells have been passaged over 135 times. Subclonal populations of positive clones show inducible virus in 1/3 of all cells tested [Hoggan, Thomas; Johnson (Brigham Young U.)].

IUDR treatment of AAV-carrier cultures increases AAV induction by adenovirus Treatment of AAV-carrier cultures with IUDR (20 $\gamma$/ml) for 18 hrs prior to adenovirus infection increases the number of cells producing AAV antigen 5-500 times. These results suggest that the AAV genome may be integrated into the host cell genome. This system provides a model for studying the occurrence and possible effects of the inducible AAV that has been found in 1% of all human embryos thus far studied [Hoggan, Thomas].

Polypeptide subunits of minute virus of mice (MVM) identified and concentra- tion of each polypeptide found to vary depending on host cell. Three distin polypeptides of MVM have been identified, and although the same three molecu weight species of these polypeptides are found in each virus preparation, relative concentration of each is different in preparations of virus grown

different host cells. These unexpected results provide insight to the basis for the wide spectrum of virus/host cell interaction seen with MVM in different hosts [Hoggan, Dieter, White].

Human papovaviruses found to share antigens with SV40. Three newly discovered human papovaviruses have been studied. All are serologically related to the simian virus 40 (SV40) and share structural (V) as well as non-virion (T) antigens. Two of these were isolated from a brain disease of humans, progressive multifocal leucoencephalopathy (PML), while the third was from the urine of a renal transplant case. Serologic evidence indicates that these are common viruses in humans, infecting 70% of the population. They may remain in "latent" or "integrated" states in the kidneys or brains of humans and become activated after immunosuppression. Their role in disease in humans, either slow, chronic infections or in neoplasia, requires further investigation [Takemoto, Mullarkey].

Interferon found to modify host RNA. Interferon exerts its antiviral action by modifying the cell's protein synthesizing system in such a way that normal cell messenger RNA's are translated normally but viral mRNA translation is inhibited. We have found that interferon treatment modifies cell mRNA population in such a way that each mRNA species migrates in gel electrophoresis more slowly than its normal counterpart. Transfer RNA (4S RNA) also is modified comparably in interferon treated cells. These data suggest that interferon treatment of cells enables the cells to distinguish between cell and viral (foreign) RNA by modifying recognition sites on the cell mRNA so that the cell mRNA differs from the subsequently entering viral RNA. Implicit in this statement is the idea that the recognition mechanism also is modified in interferon treated cells so that the "interferon type" mRNA is preferred. Perhaps the modified transfer RNA is involved in the recognition mechanism [Levy, Riley, Markoff].

Improved interferon techniques developed. Studies of the interferon system have developed a new assay system for interferon. This assay is advantageous because it allows the same procedure to be used on cells from different species and it permits a more precise definition in time of the interferon induced antiviral state. Another methodologic advance is the development of a mouse cell line which can be stimulated with Newcastle disease virus to produce very large quantities of interferon. Also a procedure was developed utilizing the genus specificity of interferon to provide a simple and quick method for determining the animal origin of certain cell cultures [Baron, Buckler, Uhlendorf, Oie].

Major role of humoral immunity shown in disseminated vaccinia. Immunosuppression studies during vaccinia virus infection of mice demonstrated that under the experimental conditions employed the mechanism by which immunosuppression enhanced virus disease was the inhibition of production of serum antibody which allowed prolonged viremia and increased seeding of the target organs by virus. Cell mediated immunity did not seem to be a factor in this study [Worthington, Baron].

Interferon inducers found to affect viral tumorigenesis. As expected, when mice inoculated with murine sarcoma virus were treated with interferon inducers before and after virus, tumor response was decreased. However, the paradoxical observation was made that with a variety of interferon inducers a single pretreatment dose enhanced tumorigenesis. This suggests a possible hazard in the use of such inducers in man [Gazdar, Baron].

Viruses of amoeba characterized and evidence indicating lysogenicity obtained Both the polyhedral and filamentous amoeba viruses have been found to contain DNA. The polyhedral virus has been shown to be an icosahedral virus with a unique folding pattern of its DNA [Hruska, Mattern, Diamond]. Normal axenically cultivated HB-301 amoebae have been cloned in the presence of antiserum All 11 of the initial clones produced virus, and all 16 recloned lines from one original clone have been shown to produce virus. This favors the concept that the virus is carried in a lysogenic state. However, no means of inducin virus has yet been found [Diamond, Hruska, Mattern].

Hamster syncytial virus found to resemble oncornaviruses. Studies on a hamst syncytial virus have shown it to resemble the oncornaviruses with respect to morphology, inhibition by actinomycin, presence of reverse transcriptase activity, and size of RNA [Hruska, Takemoto].

Neurochemical role in taste discovered. Ultrastructural studies, coupled with histochemical electron microscopy have revealed for the first time acetylcholinesterase of the pore of the rat circumvallate taste bud [Mattern, Paran This, coupled with collaborative studies employing neurochemically active dru suggests that taste is mediated through a cholinergic system [Mattern, Henkin

Further studies with an episcope have revealed evidence of some sort of contractile mechanism in the taste buds of fungiform papillae of mice. On the basis of preliminary pharmacological experiments, this contraction may be mediated through the parasympathetic system [Mattern].

Serial No. NIAID 68H
1. Viral Oncology
2. Viral Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunologic and Other Studies of Animal Leukemia Viruses.

Previous Serial Number: SAME.

Principal Investigator: Dr. M. K. Cook.

Other Investigators: Dr. W. P. Rowe, Miss Johnna Sears.

Cooperating Units: None

Man Years:

| | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 24/12 | 0 | 0 | 24/12 |
| Professional: | 12/12 | 0 | 0 | 12/12 |
| Other: | 12/12 | 0 | 0 | 12/12 |

Project Description:

Objectives: Study of the biology, immunology, and natural history of agents associated with Marek's disease and of Type I (RIF-type) and Type III (Reticuloendotheliosis - CSV) avian leukemia viruses.

Methods Employed: Tissue culture system and standard animal inoculation procedures were employed. Current methods were used to define and characterize viruses and antigens. Immunofluorescence, complement-fixation, agar-gel diffusion, immuno-electrophoresis, and neutralization tests were employed to define similarities and differences in the antigenic components of avian leukosis viruses and Marek's herpesvirus. Gradient centrifugation, column chromatography, and analysis of viral proteins and soluble antigens by polyacrylamide gel electrophoresis were used to separate antigenic components of strains of Marek's herpesvirus.

Major Findings: The failure of "soluble antigens" prepared from low tissue culture passages of field isolates of Marek's disease herpesvirus to elicit antibody response in chickens was reminiscent of the immunologic tolerance to LCM virus and to gs antigens of homotypic C-type RNA viruses of mice, chicken and cats. We found, however, that after repeated tissue culture passages, field isolates which showed conversion to type II plaque forms, as well as the established JM strains of herpes virus, did produce gs antigens

in tissue culture which were capable of eliciting antibodies in a homologous host. This conversion, after manipulation in tissue culture, of the immunologic and pathogenic capacity of an oncogenic virus and the probable acquisition of new antigens or a change or substitution of antigens represented a unique finding. The antigenic changes also were accompanied by changes in the plaque size produced in tissue culture and a change from close virus-cell-association to the free release of virus into supernatant medium.

Purification of the gs antigens from a field isolate and an established strain of Marek's herpesvirus have been accomplished, but attempts to identify the antigenic changes responsible for the shift from production of immunologic tolerance or for other changes in the characteristics of the virus have not as yet been identified.

The oncogenic properties of chick syncytial virus (CSV) reported by our laboratory earlier now have been confirmed in other laboratories and the virus has been placed in the new group of oncogenic reticuloendotheliosis viruses. Viruses of this group have been shown by us and by workers in other laboratories, to cause neurolymphomas and lympho-proliferative lesions in the peripheral nerves of chicken and other avian species.

Proposed Course of the Project: Further attempts to identify the active antigenic components of field strains and laboratory manipulated strains of Marek's herpesvirus will be made by concentration, purification, and characterization of the lipoproteins and glycoproteins of the various strains. Attempts will be made to correlate and compare the differences expected in immunologic tolerance and other laboratory traits of field isolates and established strains of virus.

The chicken probably was the first species found to have two distinct unrelate C-type viruses, avian leukemia group viruses and the antigenically distinct reticuloendotheliosis group of viruses. The gs antigen of the avian leukosis viruses has been characterized. We now will characterize the soluble gs antigen associated with CSV and/or reticuloendotheliosis virus.

Significance to Biomedical Research and the Program of the Institute: Mechanisms of cellular immunity studied by using Marek's herpesvirus strains may provide answers to the production of spontaneous tumor regression and to hypersensitivity reactions accompanying tumor production and lympho-proliferative disease. The effect of contamination by avian leukosis viruses, Marek's herpesvirus, and the reticuloendotheliosis viruses of viral vaccines prepared in tissue cultures of avian origin must be assayed. Because of their oncogeni properties, testing for the presence of Marek's herpesvirus and RE viruses should be added to safety protocols for viral vaccines.

Serial No. NIAID 71A
1. Viral Oncology
2. Viral Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies of Parvoviruses with Emphasis on the Adenovirus-Associated Viruses (AAV).

Previous Serial Number: SAME.

Principal Investigators: Dr. M. David Hoggan

Other Investigators: Dr. Wallace P. Rowe and Gunter F. Thomas.

Cooperating Units: (1) Dr. Lon R. White DB/NICHD.

(2) Dr. Kenneth I. Berns, Johns Hopkins School of Medicine, Baltimore, Maryland; Dr. Neil R. Blacklow, University Hospital, Boston, Massachusetts; Dr. F. Brent Johnson, Department of Microbiology, Brigham Young University, Provo, Utah.

Man Years:

| | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 30/12 | 0 | 2/12 | 32/12 |
| Professional: | 20/12 | 0 | 2/12 | 22/12 |
| Others: | 10/12 | 0 | 0 | 10/12 |

Project Description:

Objectives: To continue studies on the biological, immunological, biophysical, and biochemical characteristics of the parvoviruses, using in vitro and in vivo techniques in order to gain insight to their role in disease.

Methods Employed: Viruses are grown in appropriate host cell systems with appropriate radioactive labels, purified by biophysical and immunological procedures. Such purified labeled virus preparations are disrupted by various physical-chemical procedures, such as sodium dodecyl sulfate (SDS) plus reducing agents, and separated into their constituent polypeptides by polyacrylamide gel electrophoresis. The various physical properties such as molecular weight, etc., are determined and specific antibodies are prepared against the various polypeptide fractions.

Continuous cell lines such as Hep II and Detroit-6 cells are infected at high virus/cell multiplicity in the presence or absence of various helper viruses.

Such "carrier" lines are then split at regular intervals and routine cloning procedures carried out in order to determine what percentage of cells carry the genome, etc. The genome is detected by challenging carrier cells with helper virus and measuring the amount of specific viral antigen and or infectious virus produced. AAV carrier lines are checked for the presence of viral DNA using DNA/DNA hybridization technique on hydroxyapatite columns.

Major Findings: Previous studies had indicated that parvoviruses are composed of single stranded DNA and three polypeptides (one major polypeptide making up 70-85% of the total protein, and two minor polypeptides making up the remaining 15-30%), and that such polypeptides shared antigenic reactivity with the polypeptides of other members of the group. For example, VP antisera prepared against AAV-3 reacts against the polypeptides of AAV-1, 2, and 4, but not against the polypeptides of R.V., HI, MVM, and HADEN. Further, that F.A. antibody prepared against any of the rodent parvoviruses (H-1, RV, or MVM) crossreacts with other rodent parvoviruses but not AAV or HADEN virus. Recent studies on the polypeptides of MVM indicate that not only are the polypeptides of this virus quite distinct from those of the other rodent parvoviruses, but they can be quite different in different MVM preparations depending on the host cell in which they are produced. These results were unexpected in light of earlier studies which showed the host cell had little or no effect on the polypeptides of AAV.

AAV-1 carrier cultures of Detroit-6 cells have now been passaged over 135 times and still maintain the carrier state. An AAV-1 positive clone has been studied in detail and it has been possible to detect virus in about 33% of all subclones.

AAV-2 carrier cultures of Detroit-6 cells have now been established. This is particularly significant in light of the naturally occurring AAV-2 carrier state of human embryonic cells. The carrier state has been extended to another human cell line, Hep II.

Treatment of AAV-carrier cells with IUDR results in increasing the induction of AAV virus production by adenovirus helper 10 to 500 times.

Kinetic studies of antibody production against parvovirus polypeptides indicate such antibodies are produced at similar rates as antibodies produced against the whole virion.

Significance to Biomedical Research and the Program of the Institute: Since last year three new parvovirus candidates have been reported, including a human virus which causes acute nonbacterial gastroenteritis. This disease, although it is self-limiting with essentially zero mortality, has an extremely high morbidity rate, and accounts for a great deal of work time loss each year. The carrier states so frequently established in nature by many of these viruses in their natural hosts--MVM in mice, RV in rats, AAV in humans, porcine parvoviruses in hogs, with and without consequences to the host--continue to stimulate research designed to elucidate their role in human disease and embryonic development.

Proposed Course of the Project: The comparative studies of the various parvoviruses will continue.

The possible role of AAV in disease syndromes will continue to be investigated at the epidemiological level including such diseases as Reyes syndrome.

To find animal model systems for study of interrelationships between various parvoviruses.

Further studies of established in vitro carrier states.

Invited Lectures and Seminars:
Dr. M. David Hoggan:

1. Seminar, Laboratory of Viral Infection at Bureau of Biologics Standards.

2. IV Lepetit Colloquium, Cocoyoc, Mexico.

Publications:

Johnson, F. B., Blacklow N. R., and Hoggan M. D. Immunological reactivity of antisera prepared against the sodium dodecyl sulfate-treated structural polypeptides of adenovirus-associated virus. J. Virol., 9: 1017-1026, 1972.

Johnson, F. B. and Hoggan, M. D. Structural proteins of HADEN virus. Virol. 51: 129-137, 1973.

Hoggan, M. D., Thomas, Gunter F., and Johnson, F. Brent. Continuous "carriage" of adenovirus-associated virus genome in cell culture in the absence of helper adenovirus. Proceedings of the Fourth Lepetit Colloquium, North-Holland Publishing Company, Amsterdam, The Netherlands, in press.

Serial Number NIAID/NCI 71 D
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies of Mouse Leukemia Viruses

Previous Serial Number: Same

Principal Investigators: Dr. Janet W. Hartley, LVD, NIAID
Dr. Wallace P. Rowe, LVD, NIAID

Other Investigators: Dr. Sisir K. Chattopadhyay, LVD/NIAID
Dr. Douglas R. Lowy, NCI
Dr. Bernice Moll, LVD/NIAID
Dr. Marshall D. Sklar, LVD/NIAID
Dr. Natalie M. Teich, NCI

Cooperating Units: Inside NIH:
Dr. Arthur S. Levine, DCT, NCI
Dr. Nelson A. Wivel, MB, NCI
Dr. Josef Pitha, GRC, NICHD
Dr. Michael Potter, LCB, NCI
Outside NIH:
Dr. Theodore Bremner, Howard University, Washington, D.C.
Dr. Edward A. Boyse, Sloan Kettering Institute for Cancer Research, New York, New York
Dr. E. Stockert, Sloan Kettering Institute for Cancer Research, New York, New York
Dr. H. Ikeda, Sloan Kettering Institute for Cancer Research, New York, New York
Dr. H. Sato, Sloan Kettering Institute for Cancer Research New York, New York
Dr. Frank Lilly, Albert Einstein College of Medicine, New York, New York
Dr. John C. Parker, Microbiological Associates, Inc., Bethesda, Maryland
Dr. Paula M. Pitha, The Johns Hopkins School of Medicine, Baltimore, Maryland

Man Years:

| | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 158/12 | 24/12 | 0 | 182/12 |
| Professional: | 58/12 | 24/12 | 0 | 82/12 |
| Other: | 100/12 | 0/12 | 0 | 100/12 |

Project Description:

Objectives:

To utilize sensitive virus isolation and quantitation procedures for murine leukemia and sarcoma viruses in studying the natural history of infection with these agents and the biology of their growth in tissue culture.

Methods Employed:

Tissue culture procedures, using plaque and focus assays and complement fixing antigen induction; complement fixation; fluorescent antibody techniques; starch-gel electrophoresis for marker isozyme locus assays; nucleic acid hybridization.

Major Findings:

Genetics of MuLV infection: Studies on the transmission of infectious MuLV to the progeny of the high leukemia, high virus AKR mouse crossed and backcrossed to various low virus Fv-$1^n$ and Fv-$1^b$ strains have indicated that the capacity to produce infectious virus is determined by either of two unlinked chromosomal loci, designated as Akv-1 and Akv-2, which appear to be integrated genomes of the virus. One of the loci, Akv-1, has been located in linkage group I (chromosome 7), 12 map units from the Gpi-1 locus, with a gene order of c-Gpi-1-Akv-1.

That these loci represent integrated virus genetic information is suggested by the finding that the host range of the virus detected in crosses to Fv-$1^b$ strains, which are genetically restrictive for the virus from AKR, was almost always that of AKR virus.

Preliminary data on the transmission of infectious virus in matings of low virus Fv-$1^n$ mice to another high MuLV strain, $C_3$H/Fg, indicate that at least 3 virus-inducing loci are segregating in these crosses.

The Fv-1 locus of the mouse, the major determinant of the biology of MuLV infection, has been located in linkage group VIII (chromosome 4), very closely linked to the Gpd-1 locus.

Activation of MuLV by IUDR: Close correlation has been found between segregation patterns for infectious MuLV in post-natal animals and for induction of virus in embryo cells treated with 5-iododeoxyuridine (IdU). Activation of virus in individual embryo cell cultures has proved to be a sensitive technique for studying virus segregation in restrictive or low penetrance crosses.

All Fv-$1^n$ strains of mice which produce infectious virus produce only N-tropic virus, while Fv-$1^b$ strains produce B-tropic or both N- and B-tropic virus. As first reported by Aaronson et al., the virus induced by IdU or BdU treatment

of BALB/c cells (a B-type strain) has been invariably N-tropic. We are studying the host range characteristics of virus induced by IdU treatment of embryo cells of two B-type strains of mice (B10.D2 (old) and B10.Br) which in vivo produce predominantly B-tropic virus. By use of a highly sensitive wild mouse embryo cell line (see below) we can now consistently recover N-tropic virus from all B10.D2 and B10.Br embryo cultures tested to date, and B-tropic virus has been found on several occasions.

Screening of various mutagens, nucleic acid analogs, and chemical carcinogens has shown that the two thymidine analogs IdU and BdU are by far the most effective inducers of MuLV in AKR cell cultures. Studies of the mechanism of virus activation by IdU indicate that incorporation of the analog into cellular DNA is essential. Inhibition of DNA synthesis and blocking of analog incorporation prevented induction, while induction could be enhanced by simultaneous treatment with fluorodeoxyuridine, or by exposure of analog-treated cells to visible light or X-irradiation. Experiments using synchronized cell cultures demonstrate that the cells are most susceptible to IdU induction during the early part of the S phase.

Virus-specific DNA in mouse cells: Isotopically labeled single-stranded viral DNA probes have been synthesized from purified AKR and Kirsten MuLV utilizing the virion associated endogenous reverse transcriptase in the presence of actinomycin D (method of E. Scolnick). Utilizing this probe, DNA reassociation kinetics with a variety of cellular DNA's have been carried out as described originally by Britten and Kohne. Significant differences in hybridization have been noted between DNA from uninfected NIH (low leukemic) and AKR (high leukemic) mouse embryo cells. These differences have been noted with both AKR and Kirsten probes. A cloned wild mouse embryo cell line from our laboratory which has an unusually high degree of sensitivity to non-tissue culture adapted MuLV has been found to contain the smallest amount of hybridizable material of the various mouse cells tested.

Studies with polyvinylnucleotides: Studies by other investigators have established that poly A and poly U could inhibit MuLV reverse transcriptase activity as well as the replication of MuLV in tissue culture. The synthetic vinyl analogs of these compounds were also found to inhibit MuLV replication. However, kinetic and temporal studies show that these compounds do not specifically block reverse transcriptase activity in vitro and can block virus replication at later stages of the infectious cycle.

Development of cell line highly sensitive to MuLV infection: A wild mouse embryo cell line has been obtained which has the unusual property of sensitivity to both N- and B-tropic MuLV host range variants. In addition, the line is more sensitive than reference secondary mouse embryo cultures to MuLV, particularly for detecting virus in mouse tissue extracts or virus activated from embryo cell cultures. The line was developed by cloning from a feral mouse embryo cell line derived from a single fetus.

Serological classification of MuLV isolates: Virus neutralization studies have continued on isolates of MuLV from various sources. All isolates from

naturally infected inbred mice tested to date, with the exception of the agent isolated by Lerner et al. from NZB mice, have shown relationship to Gross virus. The Lerner virus, in contrast, is apparently more closely related to virus of the FMR subgroup. Further testing of viruses isolated from feral mice indicates that these viruses comprise a subgroup distinct from the Gross-AKR and FMR subgroups.

Induction of plasmacytomas by Abelson virus: In collaborative studies with Dr. Michael Potter, NCI, we have found that the Abelson virus, a lympho-sarcomagenic variant of Moloney leukemia virus, induces plasmacytomas when injected into mineral oil (pristane) treated BALB/c mice. The plasmacytomas are transplantable and produce immunoglobulins. The primary tumors arise within as short a time as four to six weeks after virus inoculation. This system opens up the possibility of inducing plasmacytomas producing antibody against a particular antigen; if this can be worked out it could revolutionize the field of antibody production.

Significance to Biomedical Research and the Program of the Institute:

Studies of the natural history of MuLV infection have now developed to a stage in which the biology of both exogenous and endogenous infection can be studied with increasing depth, with possibilities for contributing to the understanding of oncogenesis.

Establishment of chromosomal locations of integrated virus genetic material in various strains of mice may eventually permit the determination of whether inheritance of a particular viral genome is associated with development of a particular type of tumor, especially if closely linked non-viral markers could be used to detect viral genes of varying degrees of defectiveness.

The discovery of the effectiveness of the thymidine analogs in inducing production of infectious MuLV has led to wide application in studies of endogenous MuLV in mouse cells and of endogenous virus in other systems. The development of such relatively efficient systems may lead to understanding of the process of virus induction.

The development of the feral mouse embryo cell line with high sensitivity to both N- and B-tropic MuLV provides a tissue culture system superior to those used previously for detecting and quantitating MuLV. This line is of particular value in assaying field specimens and for recovering and assaying IdU-induced virus, especially in restrictive systems.

The nucleic acid hybridization studies confirm the reports of others that cellular DNA contains sequences which are complementary to viral nucleic acids. These findings, when combined with results from many other laboratories using a variety of approaches, suggest that endogenous RNA virus genomes are present in cells as DNA, probably integrated into the host genome. Furthermore, our studies suggest that mouse strains differ in the number of copies of virus genomes per cell, a situation which differs from the results reported with chicken strains.

The studies of the vinyl polymers represent an initial attempt to selectively inhibit viral replication by utilizing compounds which may inhibit the viral reverse transcriptase. Our findings demonstrate the importance of analyzing compounds in terms of their ability to inhibit viral replication, their effect on the reverse transcriptase, and their effects on non-viral cellular metabolism. It is hoped that this approach will prove useful in the testing of other potential antiviral compounds.

The system for rapid induction of plasmacytomas may be of great importance in immunology. Not only does it provide a rapid means of obtaining immunoglobuli producing tumor lines in large number, but it can greatly facilitate studies of differentiation of plasma cells and may provide a means of producing unlimited amounts of specific antibody.

Proposed Course:

Studies on the genetic transmission of MuLV and MuLV-related antigens will be continued with the aim of determining the location of the Akv-2 gene, clarifying the relationship between the known loci specifying various types of MuLV genetic information or control, and determining whether viral genes with speci fic chromosomal locations in one strain of mice are similarly located in other

Further comparisons are planned of the host range and other characteristics viruses activated from embryo cells of various inbred strains of mice. Of particular interest to us is the biology of B-tropic MuLV infection which will be explored further in both in vitro and in vivo studies.

Studies on the mechanism of IdU and BrdU activation will be continued. We plan to determine whether analog incorporation during a critical portion of the cell cycle is necessary and whether critical sites of cellular DNA must be analog-substituted for viral activation. In addition, nucleic acid hybridization studies will be carried out to determine the kinetics of viral-specific RNA production during IdU virus induction. Finally, it will be determined if IdU mediated induction is associated with viral-specific sequences going from high to low molecular weight DNA.

Further definition of the viral DNA probes will permit a more quantitative analysis of the number of viral genome copies in different mouse cells, as determined by DNA reassociation kinetics. This investigation will be coordinated with animal genetics experiments to determine if the virus inducing genes Akv-1 and Akv-2 represent structural viral genes.

Honors and Awards:

Dr. Wallace P. Rowe - Rockefeller Public Service Award

Invited Lectures:

Dr. Wallace P. Rowe - The Gustav Stern Memorial Lecture in honor of Peyton Rouse
Fifth Annual G. Burroughs Mider Lecture
13th G.H.A. Clowes Memorial Lecture

Publications:

Rowe, W.P., Lowy, D.R., Teich, N., and Hartley, J.W.: Some implications of the activation of murine leukemia virus by halogenated pyrimidines. Proc. Nat. Acad. Sci., 69: 1033-1035, 1972.

Rowe, Wallace P.: Studies of genetic transmission of murine leukemia virus by AKR mice. I. Crosses with Fv-$1^n$ strains of mice. J. Exp. Med. 136: 1272-1285, 1972.

Rowe, Wallace P. and Hartley, Janet W.: Studies of genetic transmission of murine leukemia virus by AKR mice. II. Crosses with Fv-$1^b$ strains of mice. J. Exp. Med. 136: 1286-1301, 1972.

Teich, Natalie, Lowy, Douglas R., Hartley, Janet W., and Rowe, Wallace P.: Studies of the mechanism of induction of infectious murine leukemia virus from AKR mouse embryo cell lines by 5-iododeoxyuridine and 5-bromodeoxyuridine. Virology, 51: 163-173, 1973.

Rowe, Wallace P., Hartley, Janet W., and Bremner, Theodore: Genetic mapping of a murine leukemia virus-inducing locus of AKR mice. Virology, 178: 860-862, 1972.

Rowe, Wallace P., Humphrey, James B., and Lilly, Frank: A major genetic locus affecting resistance to infection with murine leukemia viruses. III. Assignment of the Fv-1 locus to linkage group VIII of the mouse. J. Exp. Med., 127: 850-853, 1973.

Ikeda, H., Stockert, E., Rowe, W.P., Boyse, E.A., Lilly, F., Sato, H., Jacobs, S., and Old, L.J.: Relation of chromosome 4 (linkage group VIII) to MuLV-associated antigens of AKR mice. J. Exp. Med., 137: 1103-1107, 1973.

Pitha, Paula M., Teich, Natalie M., Lowy, Douglas R., and Pitha, Josef; Inhibition of murine leukemia virus replication by polyvinyluracil and polyvinyladenine. Proc. Nat. Acad. Sci. U.S., in press.

Rowe, W.P. and Sato, H.: Genetic mapping of the Fv-1 locus of the mouse. Science, in press.

Serial No. NIAID 71E
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies of Viral Antigens in Virus-induced Tumors.

Previous Serial Number: SAME.

Principal Investigators: Drs. Andrew M. Lewis, Jr. and Wallace P. Rowe.

Other Investigators: None.

Cooperating Units: Drs. Arthur Levine, Lowell Schnipper, Cephas Patch, and James Breeden (Medicine Branch, NCI); Michael Oxman, Myron Levin (Harvard Medical School); Patrick Henry, Richard Samaha (University of Missouri School of Medicine) and Paul Lebowitz, Sherman Weissman (Yale University Schoo of Medicine); Paul Berg and John Morrow (Stanford University); Thomas J. Kelly, Jr., (Johns Hopkins University); Clyde S. Crumpacker, II, (Channing Laboratory, Boston Ci Hospital).

Man Years:

| | NIAID Lab Staff | Other NIH Personnel | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 37/12 | 12/12 | 0 | 49/12 |
| Professional: | 13/12 | 0 | 0 | 13/12 |
| Other: | 24/12 | 12/12 | 0 | 36/12 |

Project Description:

Objectives: To use the adenovirus-SV40 hybrid viruses as tools to study the genetics of DNA tumor viruses; to define the role of viral genes and antigens in the oncogenic process; to study the biology of adenovirus-SV40 hybrid viruses.

Methods Employed: Tissue culture, fluorescent antibody, complement fixation, serologic epidemiology, and nucleic acid homology techniques.

Major Findings: Studies on Nondefective Adenovirus-SV40 hybrid viruses. Since 1967 we have isolated and characterized five nondefective adenovirus 2-SV40 hybrids which we have designated $Ad2^{+}ND_1$-$Ad2^{+}ND_5$. Due to their nondefective nature these agents replicate without helper viruses. They induce either early SV40 antigens and/or early SV40 RNA. The $Ad2^{+}ND_1$ hybrid replicates in both human and monkey kidney cells. It does not induce SV40 T

antigen, but does induce the SV40 U antigen, which is heat stable and serologically different from the heat labile T antigen. The SV40 specific RNA produced by the $Ad2^{+}ND_1$ hybrid is only a portion of the RNA species produced by SV40 early in its replicative cycle prior to synthesis of viral DNA.

The $Ad2^{+}ND_2$ hybrid also grows efficiently in both human and monkey cells and induces SV40 U antigen. This hybrid also induces SV40 specific transplantation immunity (TSTA) in immunized hamsters while the $Ad2^{+}ND_1$ hybrid does not. By RNA-DNA hybridization-competition the SV40 RNA induced by $Ad2^{+}ND_1$ competes with about 40% of the SV40 RNA induced by $Ad2^{+}ND_2$. Thus, in addition to inducing SV40 TSTA, the $Ad2^{+}ND_2$ virus induces more species of SV40 RNA than does the $Ad2^{+}ND_1$ hybrid.

The $Ad2^{+}ND_4$ hybrid grows in both human and monkey cells and induces SV40 T antigen in addition to SV40 TSTA and U antigen. The SV40 RNA sequences induced by $Ad2^{+}ND_4$ compete completely with all the SV40 RNA sequences induced by the other nondefective hybrids. These RNA sequences induced by $Ad2^{+}ND_4$ appear to represent all of the SV40 RNA sequences induced early by SV40 prior to the replication of viral DNA.

The $Ad2^{+}ND_3$ and $Ad2^{+}ND_5$ hybrids grow only in human cells and induce no serologically detectable SV40 antigens. The SV40 specific RNA in cells infected with $Ad2^{+}ND_3$ compete with only 40% of the SV40 RNA sequences present in cells infected with $Ad2^{+}ND_1$. However, $Ad2^{+}ND_5$ induces more species of SV40 RNA than either $Ad2^{+}ND_1$ or $Ad2^{+}ND_3$, but less than does $Ad2^{+}ND_2$ or $Ad2^{+}ND_4$.

Reconstruction experiments using DNA from the nondefective hybrids indicate that these hybrids contain progressively larger regions of the SV40 genome increasing in size from 2% of the SV40 genome in $Ad2^{+}ND_3$ to about 30% in $Ad2^{+}ND_4$. Electron-microscopic mapping techniques of heteroduplexes between nondefective hybrid and nonhybrid Ad2 DNA have confirmed the estimation of increasingly larger regions of SV40 DNA in these agents. These studies have shown that the SV40 DNA in each hybrid genome is a single linear piece inserted at a common point approximately 0.14 Ad2 map units from one end of the genome (1 map unit equals the length of the Ad2 DNA). By comparing heteroduplexes among these hybrids it was determined that the $Ad2^{+}ND_3$ contained 6% of the SV40 genome, $Ad2^{+}ND_1$ 18%, $Ad2^{+}ND_5$ 28%, $Ad2^{+}ND_2$ 32%, $Ad2^{+}ND_4$ 43%. By combining the data on antigen induction with the results of the electron microscopic study, a map was constructed relating specific regions of the SV40 genome to the induction of specific antigens. The order of these early SV40 antigen inducing regions in the SV40 DNA segments contained in the nondefective hybrids is U, TSTA and T with the U region being nearest the common end point.

By examining heteroduplexes formed between $Ad2^{+}ND_4$ DNA and SV40 DNA cleaved by $R_1$ restriction endonuclease we showed that this hybrid DNA segment corresponds to a segment on the SV40 genome between 0.11 and 0.59 SV40 fractional lengths from the $R_1$ cleavage site. This, then, is the region of the SV40 genome which induces the early SV40 antigens. During these studies a variant of the $Ad2^{+}ND_4$ called $Ad2^{+}ND_4$ DEL was found. This variant has a deletion of the SV40

DNA segment between 0.50 and 0.57 fractional lengths from the $R_1$ endonuclease cleavage point.

Studies by other laboratories have shown that RNA transcription from the genomes of lambda phage, Adenovirus 2 and SV40 occurs from both strands of the DNA. In addition, RNA transcription from the Adenovirus 2 and SV40 genome is asymmetric in that it is transcribed preferentially from 1 DNA strand during certain stages of viral replication. Such studies suggest that well defined regions must exist in the genomes of these viruses which permit the enzyme, RNA polymerase, to select the appropriate strand for transcription.

Since the nondefective hybrid $Ad2^+ND_1$ contains only 18% of the SV40 genome and induces one early SV40 antigen, it seemed likely that either the beginning or the end of the early SV40 DNA region was present in the SV40 DNA segment in the $Ad2^+ND_1$ genome. By separating the heavy and light strands of $Ad2^+ND_1$ DNA using isopycnic banding in the presence of synthetic polyribonucleotides, we showed that early SV40 RNA hybridizes with only the light $Ad2^+ND_1$ strand, whereas late SV40 RNA hybridizes equally with both strands. It has been shown by others that early SV40 RNA is transcribed from only one-third of one strand of SV40 DNA while two-thirds of the opposite strand is transcribed late in lytic infection. The reaction of late SV40 RNA with both strands of $Ad2^+ND_1$ DNA indicates that the SV40 segment within this virus contains both early and late SV40 DNA sequences suggesting the presence of a strand selection region within this small segment of SV40 DNA. Additional studies using separated strands of viral DNA suggest that all of the SV40 DNA segments within the genomes of the nondefective hybrids contain late as well as early SV40 DNA segments. The biological significance of these regions is unknown.

Other studies have shown that induction of T antigen by SV40 is sensitive to interferon, while the induction of T antigen by adenoviruses is resistant. Induction of SV40 T antigen by the defective Ad2-SV40 and Ad7-SV40 hybrids which contain covalently linked adenovirus and SV40 DNA has also been shown to be resistant to interferon. This finding suggested that the expression of SV40 genetic information in the hybrid virus genome might be under adenovirus control resulting from the synthesis of messenger RNA molecules containing both SV40 and adenovirus nucleotide sequences. The synthesis of such hybrid RNA molecules was demonstrated by subjecting RNA from cells infected with the nondefective Ad2-SV40 hybrid virus, $Ad2^+ND_4$, to sequential hybridization first with Ad2 DNA, then elution, and rehybridization with SV40 DNA. More than 30% of the SV40 RNA induced by the hybrid virus could be demonstrated to be linked to Ad2 RNA, whereas no such linkage was detected in control mixtures of Ad2 and SV40 RNA. These results indicate that this Ad2-SV40 hybrid virus induces the synthesis of hybrid RNA molecules which contain both SV40 and Ad2 nucleotide sequences.

The $Ad2^+ND_5$ hybrid contains 28% of the SV40 genome but fails to induce serologically detectable SV40 antigens. This SV40 DNA segment contains the entire SV40 DNA segment contained in the $Ad2^+ND_1$ hybrid which induces SV40 U antigen. To determine whether the SV40 RNA induced by $Ad2^+ND_5$ was functional, studies

were undertaken to compare the polyribosomal associated viral-specific RNA's induced by $Ad2^{+}ND_1$ and $Ad2^{+}ND_5$. The technique of RNA-DNA hybridization was used to detect SV40 specific RNA bound to polyribosomes. At 21 hours past infection about 33% of the SV40 specific RNA in the cytoplasm of cells infected with $Ad2^{+}ND_1$ or $Ad2^{+}ND_5$ is attached to polyribosomes. This viral specific RNA can be released from polyribosomes with EDTA. Reconstruction experiments plus sedimentation in high salt buffers indicate that the SV40 specific RNA has many properties of messenger RNA. These studies indicated that SV40 specific RNA in $Ad2^{+}ND_5$ infected cells is functional messenger RNA which is unable to induce SV40 specific U antigen. Thus the block to the induction of SV40 U antigen by $Ad2^{+}ND_5$ appears to be at the stage of translation of SV40 messenger RNA.

Nucleotide Sequence Analysis of SV40 RNA Transcribed from Nondefective Hybrids: The derivation of nucleotide sequences for deoxyribonucleic acid (DNA) oncogenic viruses is of interest for many reasons, related both to the general properties of the transcription process and messenger ribonucleic acid (RNA) in animal cells and to the specific information that might be obtained about the ordering, spacing and structure of genes for proteins directed by these viruses. SV40 is a favorable subject for sequence analysis because its biological properties have been thoroughly studied, and its DNA is transcribed asymmetrically and extensively in vitro by purified preparations of E. coli RNA nucleotidyl transferase. The availability of nondefective adenovirus-SV40 hybrid viruses containing various portions of the SV40 genome has made it possible to isolate, by nucleic acid hybridization techniques, segments of RNA corresponding to various lengths of SV40 DNA. By transcribing SV40 DNA into complementary RNA and annealing this RNA to the DNA of $Ad2^{+}ND_1$ hybrid virus, we have been able to isolate a segment of RNA transcribed from the SV40 DNA which appears to direct the formation of the SV40 U antigen. This RNA is small enough to permit extensive sequence analysis. To analyze these RNA sequences, RNA was synthesized in vitro by incubation of SV40 DNA with E. coli polymerase and either of the radioactive precursors, $\alpha$ 32 P - ATP, $\alpha$ - 32 P - CTP, $\alpha$ - 32 P - GTP, or $\alpha$ - 32 P - UTP. The resulting radioactive SV40 complementary RNA was then annealed to either $Ad2^{+}ND_1$ or $Ad2^{+}ND_3$, treated with RNase to remove unhybridized ends, and eluted. The RNA was then digested with T 1 or pancreatic RNase and mapped by two-dimensional electrophoresis and chromatography. By comparing the sequences of principal oligonucleotides from maps of transcripts prepared in 30 min syntheses and annealed to $Ad2^{+}ND_1$ or $Ad2^{+}ND_2$ with the sequences of transcripts prepared from 5-60 sec syntheses, it was shown that this set of oligonucleotides are ordered in a single continuous sequence. These results indicated that under the conditions of restricted RNA transcription used for these experiments there is a preferred site for initiation of RNA synthesis on SV40 DNA which lies within the segment of SV40 DNA common to both $Ad2^{+}ND_1$ and $Ad2^{+}ND_3$. This site was further characterized by comparing $\gamma$ - 32 P - ATP labeled transcripts from $Ad2^{+}ND_1$, $Ad2^{+}ND_3$ and adenovirus 2 following digestion with T 1 RNase. A unique initiation fragment common to $Ad2^{+}ND_1$ and $Ad2^{+}ND_3$ but missing from adenovirus 2, was detected. This fragment was found to be identical with an initiation fragment for E. coli RNA polymerase present in intact SV40 DNA.

Studies on Defective Adenovirus-SV40 Hybrid Viruses: In 1970 we described two variants of the adenovirus 2-SV40 hybrid population which differed significantly in their ability to yield SV40 virions. The $Ad2^{++}$ HEY (high efficiency yielder) produces about 10,000 times more SV40 progeny than does the $Ad2^{++}$ LEY (low efficiency yielder). These variants induce SV40 T antigen with equal efficiency, but only the high efficiency yielder ($Ad2^{++}$ HEY) induces detectable SV40 V antigen. In an attempt to understand the difference in the biological activity of these hybrids, we have determined the content and character of SV40 nucleotide sequences in infected monkey cells and in the hybrid genomes. Hybridization-competition studies indicated that the low efficiency yielder ($Ad2^{++}$ LEY) induces the full complement of late SV40 RNA sequences, but at a 10-100 fold lower concentration than does $Ad2^{++}$ HEY. When viral DNA was selectively extracted from cells and analyzed in alkaline sucrose gradients, circular forms of SV40 were detected only following $Ad2^{++}$ HEY infection. Electron microscopic study of heteroduplexes formed between hybrid DNA and Ad2 DNA showed that the $Ad2^{++}$ LEY genome contains 1.0 unit length of SV40, inserted 7% from one end of the hybrid molecule and accompanied by a 10% Ad2 deletion. $Ad2^{++}$ HEY molecules had 1.4 or 2.2 SV40 units centrally inserted, with a 35% Ad2 deletion. These results suggest that inefficient yielding of SV40 by $Ad2^{++}$ LEY is due to infrequent excision of an intact SV40 genome from the hybrid molecule.

Significance to Biomedical Research and the Program of the Institute: The recent advances in the genetic analysis of SV40 emphasize that this virus is becoming an important reagent for understanding basic cellular functions. In addition, recent studies indicate that human papovaviruses isolated from the brains of patients with progressive multifocal leukoencephalapathy (a chronic degenerative brain disease which develops in certain patients suffering from malignancies) are very closely related to SV40. As a group then SV40 and the human papovaviruses appear to offer an important new model to study the role of the viral genome in the pathogenesis of chronic diseases.

The discovery of nondefective adenovirus 2-SV40 hybrids which are in essence genetic recombinants between a common human pathogen and a simian virus which is known for its ability to produce tumors and transform cells, has produced considerable discussion as to the potentially hazardous nature of these viruses. Since it is very likely that some type of restriction will be placed upon the distribution and use of these hybrid viruses, these regulations will establish a precedent for the use and distribution of all types of potentially hazardous mutants of agents which are capable of infecting humans. The use of mutant and recombinant virus probes is likely to play a fundamental role in comprehensive studies elucidating the mechanisms of genetic control in human cells. The basic issue then in the discussion of the use of the nondefective hybrids is whether the rewards outweigh the risks inherent in this area of research. Thus the outcome of these discussions is of considerable importance to the future direction of biomedical research.

Due to the increasing emphasis being placed upon the use of animal virus mutan for vaccine development, the problems raised by the discussion of the non-

defective hybrids are of particular importance to the programs of the Institute. During this era of increased public awareness of the liabilities that scientific research can beget, the discussions of the potential risks involved in working with the nondefective hybrids should lead to a re-evaluation of the current concepts concerning the safe use of temperature sensitive mutants and recombinant viruses in the production of vaccines for human use.

Proposed Course of the Project: Studies are underway to characterize a number of lines of cells transformed by the nondefective adenovirus-SV40 hybrids and to determine if any of the SV40 DNA segments in these hybrid genomes are associated with SV40's ability to produce tumors in animals and transform cells in tissue culture. Future studies will be directed toward understanding the interactions between host cells and infecting virions which gave rise to these agents, the production of additional recombinants containing deletions in the various segments of the SV40 genome already isolated in the present hybrids, and the biological and biochemical characterization of these new recombinants to determine the effect of altering various regions of the SV40 DNA segment on the induction of SV40 functions. Due to the concern about the potential hazardous nature of these hybrids, work with infectious nondefective hybrids has been suspended. In addition to developing proposals for the distribution and safe handling of potentially hazardous agents, studies are underway to attempt to devise techniques for large scale purification of adenovirus 2 rendered noninfectious by physical or chemical treatment. Until the questions concerning the type of containment to be used to handle these agents are resolved and adequate facilities are available, no new studies will be undertaken.

Invited Addresses:

Andrew M. Lewis, Jr. - Asilomar Biohazard Conference, Pacific Grove, California.

Publications:

Patch, Cephas T., Lewis, Andrew M., Jr., and Levine, Arthur S.: Transcription control region of simian virus 40 in the adenovirus 2-simian virus 40 hybrid, $Ad2^{+}ND_{1}$. Proc. Nat. Acad. Sci., 69: 3375-3379, 1972.

Lewis, Andrew M., Jr., Levine, Arthur S., Crumpacker, Clyde S., Levin, Myron J., Samaha, Richard J., and Henry, Patrick H.: Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. V. Isolation of additional hybrids which differ in their simian virus 40-specific biological properties. J. Virol., in press.

Henry, Patrick H., Schnipper, Lowell E., Samaha, Richard J., Crumpacker, Clyde S., Lewis, Andrew M., Jr., and Levine, Arthur S.: Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. VI. Characterization of the deoxyribonucleic acid from five nondefective hybrid viruses. J. Virol., in press.

Levine, Arthur S., Levin, Myron J., Oxman, Michael N., and Lewis, Andrew M., Jr.: Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. VII. Characterization of the simian virus 40 ribonucleic acid species induced by five nondefective hybrid viruses. J. Virol., in press.

Zain, B. Sayeeda, Dhar, Ravi, Weissman, Sherman M., Lebowitz, Paul, and Lewis, Andrew M., Jr.: A preferred site for initiation of ribonucleic acid transcription by E. coli RNA polymerase within the simian virus 40 deoxyribonucleic acid segment of the nondefective adenovirus-simian virus 40 hybrid viruses $Ad2^{+}ND_1$ and $Ad2^{+}ND_3$. J. Virol., in press.

Kelly, Thomas J., Jr., and Lewis, Andrew M., Jr.: The use of nondefective adenovirus-simian virus 40 hybrids for mapping the simian virus 40 genome. J. Virol., in press.

Morrow, John F., Berg, Paul, Kelly, Thomas J., Jr., and Lewis, Andrew M., Jr.: Mapping of simian virus 40 early functions on the viral chromosome. J. Virol., in press.

Lewis, Andrew M., Jr.: Experience with SV40 and adenovirus-SV40 hybrids (including followup data). Biohazards in Virus and Cell Research, Cold Spring Harbor Press, in press.

Serial No. NIAID-71-F
1. Laboratory of Viral Diseases
2. Molecular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Interferon and Interferon Inducers

Previous Serial Number: SAME

Principal Investigator: Dr. Hilton B. Levy

Other Investigators: Dr. Louis Markoff, LVD, NIAID
Mr. Freddie Riley, LVD, NIAID

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total | 36/12 | | | 36/12 |
| Professional | 24/12 | | | 24/12 |
| Other | 12/12 | | | 12/12 |

Project Description:

Objectives

To study the interferon induced alterations in cells that enable these cells to develop resistance to virus. To determine how such alterations may activate the several other functions recently described for interferon.

Methods Employed

Many biochemical methods have been employed. Included, but not all inclusive, are spectrophotometry, single and double isotope tracing, gel electrophoresis, gradient centrifugation, paper and column chromatography, etc. In addition, various biological techniques have been employed, both in tissue culture and in animals, as indicated in the sections on results. Several extensions of the intramural program have been developed in collaborative programs through the contract branch of NIAID.

Major Findings

Some of the programs mentioned in last years report are being continued but will not be discussed here. The major activity of the laboratory during the past year was spent in studying alterations in cellular metabolism induced by treatment of cells with interferon. It will be recalled that an early explanation of the antiviral action of interferon involved observations that gross macromolecule synthesis was inhibited by treatment of uninfected cells with interferon. We were able to show that these gross alterations were attributable to impurities in the interferon preparations and were unrelated to the development of the virus resistant state. At that time, several years ago, we pointed out however, the obvious fact that some biochemical changes must take place to make the cells resistant to virus. Subsequently, our lab and others showed that interferon treatment of cells leads to a modification of ribosomes and/or ribosome associated factors such that viral messenger RNAs are poorly translated while translation of normal cell messenger RNA is affected little if at all. The question of how the cellular translation system in interferon treated cells is able to make such a subtle distinction among messenger RNA types is not understood. During the past year I have performed experiments which I think are relevant to this modified recognition system. Control cells and cells treated with interferon were exposed to radioactive uridine for a short time. Polysomes were isolated from both cultures and the messenger RNAs isolated from the polysomes by lowering the magnesium concentration. The messenger RNA on polysomes has been shown to be bound to protein and is properly referred to as messenger ribonucleoprotein (mRNP). The fact that the mRNP's were isolated from the polysomes means that they were in the process of being translated at the moment of cell disruption, as contrasted to mRNP found in the cell sap not bound to ribosomes. The mRNP's were analyzed by electrophoresis on polyacrylamide gels. After electrophoresis the gels were sliced and the distribution of RNA along the gel determined. A number of radioactive peaks were found in the mRNPs from both the control cells and the interferon treated cells. The shapes of the two curves were quite similar. However, uniformly, the components from the interferon treated cells moved more slowly than did the corresponding control components. These differences in migration speed could be attributable either to the RNA or the protein in the mRNP or to both. To determine which component was responsible, mRNA was extracted from the mRNP by phenol. The mRNA from both control and interferon treated cultures again resolved into a number of discrete peaks and again the interfer components moved more slowly than did the controls. The speed

movement of RNA molecules on polyacrylamide gels is a function of the molecular weight and of the charge of the molecules. Running the mRNAs on gels in the presence of sodium dodecyl sulfate eliminates charge differences. Again, the mRNA components from the interferon treated cells moved more slowly than did the corresponding controls. Therefore, it can be concluded that the mRNAs from cells treated with interferon preparations are larger than normal messenger RNAs. The difference in migration speed would suggest that they are larger by perhaps 10 nucleotides. This effect was seen in many experiments. That the effect is attributable to the interferon in the preparations and not to impurities is indicated by several lines of evidence; (1) The same effect is noted with interferons both from mouse tissue culture or mouse serum. These interferons be expected to have different impurities; (2) experiments were done with both mouse L cells and mouse embryo cells. While the shapes of the curves with primary mouse embryo cells were quite different from those of L cells the interferon effect was present with both groups. (3) The phenomenon shows the species specificity which is part of the operational definition of interferon. Rabbit interferon induces the same type of effect on rabbit tissue culture cells but not on mouse cells, conversely mouse interferon, while active on mouse cells, has no effect on rabbit cells. (4) With 5 units of interferon there is definite effect on virus growth, and the described biochemical phenomenon can be observed, while with less than 1 unit of interferon no inhibition of virus was observed and the biochemical alterations described are not noted. (5) If interferon is present for only two hours, a time too short to induce the virus resistant state, the alteration in messenger RNA is not seen.

The effect is restricted to the messenger RNA fraction and the 4S transfer RNA fraction. Ribosomal RNA does not appear to be altered nor does the RNA extracted from the nucleus. There may be some alteration in the messenger RNA patterns in the cell sap; i.e. that messenger which is not bound to ribosomes.

Two questions immediately present themselves. (1) What is the physical chemical change that is taking place in the messenger RNAs and (2) what is the biological significance of the change. (1) It appears most unlikely that there is a change in the information content of the messenger RNAs. First of all if there was such an informational change in all the messenger RNAs one could expect that there would be profound alterations in cell behavior and there would be toxicity. No such effects were noted. Secondly, if information changes were taking place, it would be surprising to find the two curves so identical except for migration speed. Therefore, it is tempting to think that

there is a uniform addition of some piece to all the messenger RNA molecules. An example of such a possibility would be an alteration in the length of the poly A of all the messenger RNA species. Preliminary experiments are consistent with this possibility.

The biological significance of these findings--It may be that cells treated with interferon develop the ability to distinguish between their own messenger RNA and foreign messenger RNA by modifying their own messenger RNAs so that they are different from the foreign (viral) RNA. This implies that not only is the cellular messenger RNA modified but that cellular mechanisms that recognize endogenous messenger RNAs be modified so as to prefer the new modified messenger RNA to the older type. Such a mechanism would provide the molecular basis for selective post transcriptional control of protein synthesis--an area of much interest in molecular biology.

Significance to Biomedical Research and the Program of the Institute

The immediate significance of this observation is that for the first time an important effect of interferon on cell molecular biology has been shown. Previously the earliest effect of interferon that had been demonstrated was on the interaction between the virus and the cell. The present results may explain how such interactions are altered. The broader implications of these observations is that the cell has built in mechanisms whereby it may alter selectively recognition sites in messenger RNA molecules.

Proposed Course of the Project

Experiments for the forthcoming year will be along three lines. (1) To investigate the molecular nature of the alteration in messenger RNA from interferon treated cells. (2) To test whether ribosomes or associated factors from interferon treated cells are able to recognize "interferon type" messenger RNA better than they recognize normal control type messenger RNA and (3) to determine how the cells alter the messenger RNA after interferon treatment. For example, is there the synthesis of a new enzyme which adds additional nucleotides into existing RNA?

Honors and Awards:

I was asked to write review articles on interferon for (1) an American Chemical Scoeity Monograph and (2) an Italian Encyclopedia.

Publications:

Levy, H. B.: Interferon. In American Chemical Society Monograph, 1973, in press.

Levy, H. B., Duenwald, J., and Buckler, C. E.: Chlorite-oxidized amylose as an adjuvant for interferon production. Infect. and Immun. 7: 457-460, 1973.

Levy, H. B., and Wheeler, J.: Induction of interferon by input virion. Arch. fur. de. gesamte virusforsch., 1973, in press.

Braun, W., and Levy, H. B.: Interferon preparations as modifiers of immune responses. Proc. Soc. Exp. Biol. Med. 141: 769-773, 1972.

Margolis, S. A., Oie, H., and Levy, H. B.: The effect of interferon, interferon inducers or interferon induced virus resistance on subsequent interferon production. J. Gen. Virol. 15: 119-128, 1972.

Homan, E. R., Zendzian, R. P., Schott, L. D., Levy, H. B., and Adamson, R. H.: Studies on poly I:C toxicity in experimental animals. Toxicol. Appl. Pharmacol. 23: 579-588, 1972.

Worthington, M., Levy, H. B., and Rice, J.: Late therapy of an arbovirus encephalitis in mice with interferon and interferon stimulators. Proc. Soc. Exp. Biol. Med., 1973, in press.

Serial No. NIAID-71-G
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structure and Replication of Viruses

Previous Serial Number: SAME

Principal Investigator: Dr. C. F. T. Mattern

Other Investigators: Dr. Jerome F. Hruska, LVD, NIAID
Mr. Wendell A. Daniel, LVD, NIAID
Dr. Herman G. duBuy, LVD, NIAID
Dr. Kenneth K. Takemoto, LVD, NIAID

Cooperating Units: Dr. Louis S. Diamond, LPD, NIAID, 122-G1
Dr. Ruth Pertel, LPD, NIAID, 122-G1
Dr. Shigeko Nomura, VLL, NCI-4850
Dr. Robert Bassin, VLL, NCI-4848
Dr. Paul Peebles, VLL, NCI-4835
Dr. Bernice Eddy, Biologics Control

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total | 27/12 | | | 27/12 |
| Professional | 24/12 | | | 24/12 |
| Other | 3/12 | | | 3/12 |

Project Description:

Objectives

The major emphasis of the project falls into three areas: (1) the continued study of biological, physical and chemical properties of viruses of Entamoeba histolytica and their possible relationship to human disease. (2) Collaborative studies on the potential of murine sarcoma virus-transformed cells to revert an

retransform, to produce defective C-particles, reverse transcriptase and gs antigen, their rescuability with MuLV, and their inducibility with IUdR. (3) Collaborative study on the identification and comparison of human herpes viruses, types 1 and 2 and others isolated from malignancies of man, but of unknown significance.

Methods Employed

These studies involve high resolution electron microscopy of viruses and sections of infected and normal cells employing a variety of stains. A variety of chemical and physical techniques are utilized in the purification and study of the biological and chemical properties of viruses.

Major Findings

(1) Ameba virus: Because of extraordinary difficulty in freeing the mature polyhedral virus from debris of lysed amoebae, indirect methods have employed to determine the type of nucleic acid contained by these agents. The filamentous agent from ABRM amoebae produces intranuclear inclusions of filaments which stain yellow-green with acridine orange. Further, virus replication is virtually halted by doses of IUdR, BUdR and cytosine arabinoside which permit viability in more than 70% of amoebae. Thus the filamentous agent appears to contain double stranded DNA.

The polyhedral virus of HB-301 amoebae is likewise inhibited by IUdR, BUdR and cytosine arabinoside. However, since no specific intracellular inclusions have been seen by light or electron microscopy, it was not unexpected that no identification of its nucleic acid was possible by acridine orange. However, extraction of $^{3}H$ thymidine labelled DNA revealed that infected cultures produced an unusual quantity of DNA which was of the same buoyant density in CsCl as that from normal amoebae, but of smaller size (velocity sedimentation). Hybridization studies identified the DNA as a new species not found in detectable quantity in normal amoebae. Thus this polyhedral virus also appeared to contain double stranded DNA.

The morphology of the polyhedral virus was studied by electron microscopy. The identification of 5-, 3-, and 2-fold axes of rotational symmetry verify its being an icosahedral virus. Further, its DNA appears to have a unique folding arrangement with 3 axes of 2-fold rotoreflection symmetry. A

manuscript has been prepared and is ready for submission for publication (Hruska, Mattern, Diamond).

(2) Cloning experiments in HB-301 amoebae have been hampered by a 6 month period in which satisfactory media for amoebal cultivation were not available. Nonetheless, in the first cloning experiment for which it is necessary to deaxenize the amoebae, 11 clones were established and tested for viral activity on multiple occasions. Some clones were reaxenized, other carried as monaxenic cultures with trypanosomes. All of the 11 clones have convincingly shown virus on at least one occasion.

Clone 1, which was consistently positive, was recloned in antiserum; 16 of its clones have been established and have been shown to produce a virus which is lytic for HK-9 amoebae. However, the clones do not uniformly produce the relatively high titer of virus produced by the uncloned parental HB-301 amoebae (Diamond, Hruska, Mattern).

(3) Collaborative studies with investigators of the NCI.

(a) A human breast tumor cell line was established in continuous culture and examined extensively but unsuccessfully by electron microscopy for recognizable virus particles both with and without BUdR treatment (Bassin et al.).

(b) Examination of a human amnion cell line transformed by murine sarcoma virus with a feline leukemia virus coat has failed to reveal recognizable defective sarcoma particles in these reverse transcriptase negative cells (Peebles et al., unpublished). The potential of employing these transformed cells which do not produce viral particles in rescue experiments with potential human C-particles is under study by the NCI group (Peebles et al.).

(c) Murine sarcoma-transformed 3T3 cells (FG-10) contain only the sarcoma genome and are designated S+L- (sarcoma+, leukemia-). Spontaneous and induced reversion of these cells to flat (untransformed) as well as spontaneous retransformation are under intensive study in the NCI. Various markers (reverse transcriptase, inducibility, rescuability, gs antigen, chromosome morphology and numbers, cell morphology, and production of C-particles) are being followed and their interrelations studied (Nomura et al.).

(4) Studies of a hamster synctium-forming virus have demonstrated the following: (1) the virus somewhat resembles

C-type particles morphologically; (2) its replication is inhibited by actinomycin D; (3) it possesses an RNA-dependent DNA polymerase; and (4) it possesses ribose nucleic acid of approximately 60S which is denatured by heat to 18S. Thus, this virus would appear to be a virus of the "oncornavirus" group although attempts by others have failed to demonstrate its oncogenic potential or a common "interspecies" gs-3 antigen (manuscript in preparation Hruska and Takemoto).

Significance to Biomedical Research and the Program of the Institute

These studies are intended to contribute to our knowledge of the processes by which both RNA and DNA viruses replicate. The ultimate goal, which is finding means of interrupting the viral replication cycle, will undoubtedly require approaches involving multiple disciplines, including such electron microscopic studies.

(1) The possible medical significance of protozoal viruses is being investigated.

(2) The nature of the "carrier" culture of viruses in E. histolytica is being investigated to see if this is a lysogenic system or a more conventional carrier state.

(3) Studies on the mechanisms of murine sarcoma transformation and reversion of cells may provide clues as to basic processes in human malignancies.

Proposed Course of the Project

Continued intensive study of all phases of the new amoebal viruses is planned in collaborative with LPD. It is also planned to further explore the possibility that other human protozoal pathogens may have indigenous viruses. Continued studies on a possible relationship between viruses and pathogenicity are in progress.

Publications:

Hruska, J. F., Mattern, C. F. T., Diamond, L. S., and Keister, D. B.: Viruses of Entamoeba histolytica. III. Properties of the polyhedral virus of the HB-301 strain. J. Virol. 11: 129-136, 1973.

Bassin, R. H., Plata, E. J., Gerwin, B. I., Mattern, C. F. T., Haapala, D. K., and Chu, E. W.: Isolation of a continuous epitheliod cell line, HBI-3, from human breast carcinoma. Proc. Soc. Exp. Biol. Med. 141: 673-680, 1972.

Nomura, S., Fischinger, P. J., Mattern, C. F. T., Peebles, P. T., Bassin, R. H., and Friedman, G. P.: Revertents of mouse cells transformed by murine sarcoma virus. I. Characterization of flat and transformed sublines without a rescuable murine sarcoma virus. Virology 50: 51-64, 1972.

Hruska, J. F., Mattern, C. F. T., and Diamond, L. S.: Viruses of Entamoeba histolytica. IV. Studies on the nucleic acids of filamentous and polyhedral viruses. J. Virol, 1973, in press.

Fabisch, Ph. B., Takemoto, K. K., and Hruska, J. F.: Characteristics of a hamster "foamy" virus. Abstr. Ann. Mtg. Amer. Soc. Microbiol. 365: 155, 1973.

Serial No. NIAID-71-G1
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biological Ultrastructure

Previous Serial Number: SAME

Principal Investigator: Dr. C. F. T. Mattern

Other Investigator: Mr. Wendell A. Daniel
Dr. H. G. duBuy, LVD, NIAID

Cooperating Units: Dr. Bronislaw M. Honigberg, Dept. Zoology, University of Massachusetts
Dr. Robert I. Henkin, Clinical Endocrinology Branch, NHLI 220(C)
Dr. Ruth Pertel, LPD, NIAID, 122-J

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total | 18/12 | | 12/12 | 30/12 |
| Professional | 12/12 | | 12/12 | 24/12 |
| Other | 6/12 | | | 6/12 |

Project Description:

Objectives

To study the ultrastructure of protozoa, metazoa, and vertebrates with particular emphasis on the correlation of ultrastructure and function. Organisms and organs under study are selected both to study phylogenetic kinships and certain details of embryogenesis.

Methods Employed

High resolution electron microscopic observations are made in sectioned cells stained by conventional methods and specific histochemical reactions and by autoradiographic electron microscopy in order to elucidate the chemical composition and function of select organelles.

Major Findings

(1) By conventional thin section electron microscopy we have examined the ultrastructure of Dientamoeba fragilis and found this organism more closely related to trichomonads than amoeba. The organism is highly unusual in that it is binucleate and maintains a permanent microtubule spindle throughout most of its cycle which represents arrested telophase (Honigberg, Camp, Mattern). The organism is of great potential interest in the study of cell division in protozoa.

(2) Continued studies on the ultrastructure of vertebrate and invertebrate contact chemoreceptors has led to an hypothesis regarding the function of the multiple cell types of the human taste bud. Predicted on the basis of this hypothesis was the presence of acetylcholine and acetylcholinesterase at the pore of the circumvallate taste buds. Our preliminary findings on rat taste buds have uncovered the first evidence that taste is mediated through a cholinergic system. We have found acetylcholinesterase distributed along the finger-like projections of the taste bud cells within the taste pore. Appropriate controls, including esserine and BW have blocked this specific histochemical reaction. We have been unable to date, to demonstrate the presence of acetylcholine by autoradiography. However collaborative studies on the taste response in human volunteers has shown that succinylcholine (an acetylcholine inhibitor by competion) drastically blocks all qualities of taste, bitter, sour, salty, and sweet (Henkin,Mattern). The effect of a variety of agents on the process of taste reception is being quantitatively studied.

A second prediction was that the type II cell with a helical bundle of tonofilaments might function in contraction and relaxation permitting protrusion and retraction of the cell projections of the taste pore. By means of an episcope, a TV camera, and a video tape recorder we have demonstrated an outpouring of fluid from the pore of the mouse fungiform papilla as the result of a noxious stimulus from HCl vapor. This is

compatible with the proposed Type II cell function, in the absence of any other structure which might conceivably contract and relax. Furthermore preliminary studies with neurochemically active drugs suggests this effect, also present in the amputated tongue, is under some parasympathetic control, being inhibited by topical reserpine, a catecholamine depleting drug (Mattern).

Significance to Biomedical Research and the Program of the Institute

The taste apparatus provides a unique system for the study of sensory function since the taste cells are in contact with the external (oral) environment and can be treated locally as well as readily biopsied. We have provided the first clues as to the mechanism of contact chemoreception by demonstrating the presence of acetylcholinesterase in the taste pore and the inhibition of taste by succinylcholine. Further study is needed to evaluate the precise roles of the 3 or possibly 4 types of cell found in the mammalian taste bud.

Proposed Course of the Project

These comparative ultrastructural studies of the anatomy of protozoa, metazoa, vertebrate cells and organs will be continued. They will be correlated with physiological studies of vertebrate and invertebrate organs.

Publications:

Mattern, C. F. T., Honigberg, B. M., and Daniel, W. A.: Fine-structural changes associated with pseudocyst formation in Trichomitus batrachorun. J. Protozool.20: 222-229, 1973.

Honigberg, B. M., Daniel, W. A., and Mattern, C. F. T.: Fine structure of Trichomitus batrachorum (Perty). J. Protozool. 19: 446-453, 1972.

Serial No. NIAID-71-H
1. Laboratory of Viral Diseases
2. Cellular Vrilogy Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Host defenses against viral infection: Interferon and Immunity

Previous Serial Number: SAME

Principal Investigator: Dr. Samuel Baron

Other Investigators: Dr. Michael Worthington, LVD, NIAID
Charles E. Buckler, LVD, NIAID

Cooperating Units: Dr. E. Chowchuvech, New York Med. College
Dr. A. Gazdar, NIH, NCI 4824
Dr. A. Rabson, NIH, NCI 532
Dr. H. Oie, NIH, NCI
Dr. E. Zimmerman, Microbiological Associates

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total | 48/12 | | 4/12 | 52/12 |
| Professional | 36/12 | | | 36/12 |
| Other | 12/12 | | 4/12 | 16/12 |

Contract Support:

| Contract Number | Project Officer | Contractor | Contractor's Project Director | Annual Funding Level |
|---|---|---|---|---|
| NIH 72-C-644CC | Dr. S. Baron | North American Biologicals, Inc. | Dr. R.R. Rafajko | $73,000 |
| PH 43-68-1266 | Dr. S. Baron | New York Medical College | Dr. M. Galin | $60,000 |

Project Description:

Objectives

Studies were conducted to help define some of the mechanisms governing the host defenses (interferon and immunity) during viral infections at the cellular and whole animal level with a view toward control of human viral infections.

Methods Employed

Cell cultures from a variety of animal species including man were utilized to study the interferon system in vitro. Mice, rats, and rabbits were used to study the effect of the host defenses during viral, toxoplasma and tumor cell challenges. The infectious agents employed included vaccinia virus, mouse sarcoma virus, group A arboviruses, Newcastle disease virus, scrapie agent, and toxoplasma. The drugs employed included the interferon inducers, polyinosinic.polycytidylic acid and tilorone and the immunosuppressive agent Cytoxan.

Major Findings

1. Interferon. Improved assays for a variety of interferons, based on the inhibition of yield of viral hemagglutinin have been developed. These assays are advantageous because they combine the characteristics of rapidity, simplicity, reliability, sensitivity, and inhibition of viral multiplication during a single cycle of virus growth.

During a comparative study of the interferon system in normal and virus-transformed murine (243) cells, a high interferon producing line of transformed murine cells was identified and studied. Under optimal stimulatory conditions this cell line regularly produces 50,000 units of mouse interferon per ml of tissue culture fluid.

The genus specificity of the antiviral action of interferon was utilized to develop a typing system for cell cultures derived from different animals. In this way, a rapid and simple distinction could be made between primate, mouse, rat, hamster, and other cell types.

Experiments were undertaken to determine the effect of long term administration of a potent interferon stimulator, polyinosinic.polycytidylic acid, on the development of scrapie in mice challenged peripherally with low doses of the scrapie agent.

Scrapie infection was not influenced by continuous treatment with polyinosinic.polycytidylic acid, even when a low challenge dose of the scrapie agent was injected peripherally.

Since interferon has been noted to protect certain tissue cultures against toxoplasma gondii, in vivo, ocular protection experiments using a potent interferon inducer were undertaken. Therapy with polyinosinic.polycytidylic acid initiated two and one-half hours prior to infection provided a mild degree of protection against toxoplasma gondii of the Beverley strain. However, no protection was noted when the RH strain of toxoplasma gondii was used.

In contrast to the reported inhibition of tumors by interferon and its inducers under conditions of continued treatment, pretreatment with a single dose of several interferon inducers enhanced virus induced sarcomas and leukemias in mice and rats. Enhancement was dependent on multiple factors including the animal strain, age of mice and time of treatment. The strain dependent effect of polyinosinic.polycytidylic acid could not be explained by differences in the amount of circulating interferon induced, the extent and duration of the hyporesponsive period to interferon induction or in the level of the induced antiviral state. Also there was no correlation between the strain-dependent adjuvant properties of polyinosinic.polycytidylic acid on humoral immunity and its effect on tumor induction.

2. Immune mechanisms. A study was undertaken to help define the role of cellular immunity, humoral antibody, and interferon in recovery from primary systemic vaccinia virus infection in mice. Administration of Cytoxan in doses capable of inhibiting both humoral and cellular immunity markedly potentiated primary systemic vaccinia virus infection in mice. Immunosuppressed mice did not form neutralizing antibody to vaccinia virus and had a prolonged and more severe viremia than non-immunosuppressed control mice. Passive transfer of physiologic amounts of neutralizing antibody late in the course of infection, at a time when non-immunosuppressed mice had similar levels of serum antibody, largely reversed the effect of Cytoxan on vaccinia virus infection. Transfer of 100 million immune spleen cells was much less effective than antibody in reversing the effect of Cytoxan on vaccinia virus infection and mice receiving these cells did make some antibody. Serum interferon levels were not affected by Cytoxan. The results suggest an essential role for humoral antibody but not for cellular immunity in recovery from primary vaccinia virus infection in the mouse.

Significance to Biomedical Research and the Program of the Institute

The newly developed interferon assay provides a technically simple system for comparison of the interferon system among a large variety of animal species. It also utilizes a single cycle of virus growth which permits a more precise definition in time of the interferon-induced antiviral state. The ability to produce large quantities of murine interferon in 243 cells permits studies of the mechanism of action of interferon and its protective effects against viral infections which were not possible previously. The cell typing procedure utilizing the genus specificity of interferon provides a simple and quick method for determining the origin of certain cell cultures with applications in research and production laboratories.

The lack of effect of the interferon system on the scrapie agent and the inability of the scrapie agent to induce interferon indicates that this "virus" is the first such agent which does not interact at all with the interferon system. The lack of protection by interferon against one of the two strains of toxoplasma studied raises the possibility that the previous reports of interferon activity against toxoplasma may be due to non-interferon components of the treatment preparation. Enhancement of viral oncogenesis by pretreatment with a single dose of a variety of interferon inducers suggests that the enhancement is due to interferon and that any such studies in man should guard against conditions which would enhance tumor growth rather than retard it.

There are reports in the literature which propose that poxvirus infections in general are resisted by cell mediated immunity rather than by antibody. The present study in mice clearly demonstrates that under the conditions of our study, antibody and not cell mediated immunity was a critical factor in terminating the viremia and preventing lethal viral spread to target tissues. Immunological studies designed to control human poxvirus infections must utilize this information.

Proposed Course of the Project

The newly developed interferon assay will be used routinely in our studies of the interferon system and is being used in other laboratories as well. The system for producing high levels of mouse interferon has been distributed world wide to other laboratories and is being used in our laboratory to produce large amounts of murine interferon to study the mechanisms of the

interferon system and methods to control viral infections and tumors. The studies of the apparent lack of effect of interferon on the toxoplasma infection will be extended to an in depth study in tissue culture to determine whether interferon affects toxoplasma multiplication. The study on immunosuppression to define the relative roles of interferon, antibody and cell mediated immunity will be extended to other viral infections and it will utilize other immunosuppressive agents.

Honors and Awards (Dr. Baron):

Associate Editor, American Journal of Epidemiology

Associate Editor, Infection and Immunity

Associate Editor, International Journal of Cancer

Member of Committees for American Association of Immunologists, and American Society for Microbiology

Member of the Board of Director of the American Type Culture Collection

Invited lectures at Universities, Research Institutes, and Symposia

Publications:

Zimmerman, E. M., Freeman, A. E., Price, P. J., Holbrook, Z., and Uhlendorf, C. P.: A simple interferon assay as an adjunct for determining the genus of origin of cell cultures. In Vitro 8: 85-90, 1972.

Gazdar, A. F., Stineberg, A. D., Spahn, G. F., and Baron, S.: Interferon Inducers: Enhancement of viral oncogenesis in mice and rats. Proc. Soc. Exp. Biol. Med. 139: 1132-1137, 1972.

Hill, D. A., Baron, S., Perkins, J. C., Worthington, M., VanKirk, J. E., Mills, J., Kapikian, A. Z., and Chanock, R. M.: Evaluation of an interferon inducer in viral respiratory diseases. J. Amer. Med. Assoc. 219: 1179-1184, 1972.

Baron, S.: Interferon. In Rosenbaum, M. J., and Peckinpaugh, R. O. (eds.): Prospectives for Control of Acute Respiratory and Meningococcal Diseases. Great Lakes, Ill. Naval Medical Research Unit No. 4, 1971, pp.138-145.

Worthington, M., Rabson, A. S., and Baron, S.: Mechanism of recovery from systemic vaccinia virus infection. I. The effects of cyclophosphamide. J. Exp. Med. 136: 277-290, 1972.

Oie, H. K., Buckler, C. E., Uhlendorf, C. P., Hill, D. A., and Baron, S.: Improved assays for a variety of interferons. Proc. Soc. Exp. Biol. Med. 140: 1178-1181, 1972.

Baron, S.: Interferon. Ency. of Chem. 3rd ed., 1972, 1448-1449, 1972.

Baron, S.: Role of the interferon system during arbovirus infections. In Proc. Conf. Pathogenesis Arboviral Infections, Mild Arboviral Syndromes, vol. I., Honolulu, U. S. Japan Cooperative Med. Sci. Program, 1971.

Notkins, A. L., Bankowski, Baron, S., et al. Workshop on the treatment and prevention of herpes simplex virus infections. J. Infect. Dis. 127: 117-119, 1973.

Oie, H. K., Gazdar, A. F., Buckler, C. E., Baron, S.: High interferon producing line of transformed murine cells. J. Gen. Virol. 17: 107-109, 1972.

Chowchuvech, E., Weissenbacher, M., Schmunis, G., Sawicki, L., Galin, M. A., and Baron, S.: The influence of polyinosinic.polycytidylic acid complex on experimental acute toxoplasmic retinochoroiditis in rabbits. Investigative Ophthal. 11: 182-188, 1972.

Worthington, M.: Interferon system in mice infected with the scrapie agent. Infect. and Immun. 6: 643-645, 1972.

Glasgow, L. A., Galasso, G. J., Baron, S., et al.: Isoprinosine: Lack of antiviral activity in experimental model infections. J. Infect. Dis. 126: 162-169, 1972.

Serial No. NIAID-71-I
1. Laboratory of Viral Diseases
2. Molecular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The interaction between latent and slow viruses, especially the Lactic Dehydrogenase Virus (LDV) and the subcellular elements of the host cell (mouse macrophages for LDV). Use of the LDV-modified macrophages model for the study of role of macrophages in the defense mechanisms of the host.

Previous Serial Number: SAME

Principal Investigator: Dr. Herman G. duBuy

Other Investigators: Dr. C. F. T. Mattern, LVD, NIAID
Dr. Samuel Baron, LVD, NIAID

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total | 24/12 | | | 24/12 |
| Professional | 12/12 | | | 12/12 |
| Other | 12/12 | | | 12/12 |

Project Description:

Objectives

Study of the factors, both in vivo and in vitro, which determine whether a virus kills the host, becomes chronic, or is eliminated, and of factors which modify the outcome of the virus infection: interferon and its inducers, humoral antibody, and cellular defense factors, especially those of macrophages, the target cells of LDV.

Methods Employed

The murine Lactic Dehydrogenase virus is used as a model for other chronic viremias and latent infections because it is an RNA membrane virus which multiplies exclusively in mouse macrophages, leading to a chronic viremia without overt morbidity of the host. Subprojects have been developed which allow the use of this model for the determination of in vivo and in vitro changes of macrophages functions (induction of interferon by viruses multiplying in macrophages, action of exogenous interferon or its inducers on the multiplication of these viruses, clearance of i.v. introduced viruses, or interferon, or serum enzymes, etc.).

Major Findings

The study on the sensitivity of LDV to interferon as compared to that of two other viruses, mentioned in the previous annual report, was extended to cover 4 phases. These are: (1) The in vivo induction of interferon by LDV. (2) The in vivo action of exogenous interferon on LDV, in which the interferon is administered either prophylactically, or therapeutically by administration of interferon in single or multiple doses at different times after LDV inoculation. (3) The in vitro induction of interferon by LDV in macrophage cultures, and (4) the in vitro action of exogenous interferon, administered to macrophage cultures either prophylactically or therapeutically. The results show that LDV is a poor interferon inducer, with no demonstrable interferon produced in vitro, it is also relatively insensitive to interferon. This relative insensitivity was demonstrable both in vivo and in tissue culture. These results are incorporated in a manuscript to be presented for publication (a preliminary report appeared in the ISM). No treatment was found to cure the infection, due to an interferon resistant fraction, also reported by others. The nature of this resistance is under further investigation.

A similar four-phasic study on the role of the interferon inducer poly I.poly C is nearly finished with similar results.

A considerable effort was made to determine whether macrophages of other origins were comparable to peritoneal macrophages in their reaction to LDV-infection and interferon production. Particularly, the cultivation of spleen macrophages under interferon test conditions, as used for the peritoneal macrophages, has not yet been successful.

Significance to Biomedical Research and the Program of the Institute

The study of the chronic viremia caused by LDV contributes to the understanding of other chronic or latent viral infections. Because of the specific involvement of macrophages in the macrophage-LDV model, it may further clarify the role of macrophages in resistance to chronic viral diseases.

Proposed Course of the Project

Continuation of the studies to eliminate the chronic LDV viremia or to prevent the LDV replication in macrophage cultures by using interferon or interferon inducers with differing properties (e.g. M.I.F.-interferon), or by agents which change macrophage functions.

Expansion of the studies on the interaction between LDV and macrophages, obtained from loci other than intraperitoneal.

Publications:

duBuy, H. G.: Effect of local or distal therapeutic treatment with polyinosinic.polycytidylic acid on survival of mice with Ehrlich Carcinoma. J. Natl. Cancer Inst., 48:1525-1527, 1972.

Serial No. NIAID-71-J
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Oncogenic and "Slow" Viruses

Previous Serial Number: SAME

Principal Investigator: Dr. Kenneth K. Takemoto

Other Investigator: Dr. Michael F. Mullarkey, LVD, NIAID
Dr. J. Hruska, LVD, NIAID

Cooperating Units: Dr. Malcolm A. Martin, LBV, NIAID-80A
Dr. George Fareed, LBV, NIAID-84C
Dr. George Khoury, LBV, NIAID-80A
Dr. T. Aoki, NCI 4864
Dr. Daniel Nathans, Johns Hopkins University Medical School, Baltimore, Md.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total | 36/12 | | | 36/12 |
| Professional | 24/12 | | | 24/12 |
| Other | 12/12 | | | 12/12 |

Project Description:

Objectives

(1) To characterize biologically and biochemically heretofore unclassified newly discovered viruses which cause slow and chronic infections, and to examine their relationship to oncogenic viruses; (2) To study tumor viruses and tumor cells from a broad standpoint in order to elucidate mechanisms involved in viral carcinogenesis.

Methods Employed

Standard cell culture and virologic techniques are used.

Major Findings

During the past 3 years, an extensive study was made on RNA-containing viruses which are responsible for slow infections of sheep. These viruses belonging to the visna-maedi-progressive pneumonia complex were shown to have properties which are common to the oncogenic RNA-containing viruses. Based on these studies, certain ideas have evolved which may perhaps provide explanations concerning mechanisms of slow viral infections. One hypothesis which we favor is that RNA as well as DNA-containing viruses which, like tumor viruses, can integrate their genomes into host cells may be causative agents of slow viral infections.

Recently, the isolation of human papovaviruses was reported from 3 different laboratories. Three of the isolates were from cases of progressive multifocal leukoencephalopathy (PML), a slow demyelinating disease of humans. The fourth was from the urine of an immunosuppressed patient who had received a kidney transplant. We have obtained all of the isolates and have made preliminary characterization of the viruses.

1. DAR virus. We have confirmed Weiner's original reports that the DAR virus from a case of PML is indistinguishable from SV40 by serologic tests. Molecular hybridization studies also failed to provide any evidence of differences between the DNA's of the 2 viruses.

However, biological as well as biochemical differences have been noted. The virus appears to have a different replication pattern from SV40, the major difference being the production of large amounts of defective particles which have a major deletion (20% of the total genome) and therefore cannot replicate to form complete particles. However, these defective particles which do not induce T-antigens are tumorigenic for hamsters and at the present time (5 months after inoculation), tumors have appeared at the site of inoculation in 5 out of 28 hamsters which were infected at birth. These tumors are being established in culture and examined for the presence of T-antigen, viral DNA and RNA, and rescueable virus.

2. BK virus. BK virus was isolated from the urine of a patient who had undergone renal transplant. Our studies have confirmed the original observation that this virus is related to SV40, as determined by neutralization and fluorescent antibody tests. It also induces the same T-antigen as SV40.

Serologic studies proved that this virus was present in humans prior to the widespread use of poliovirus vaccines, thus ruling out the possibility that BK is a monkey virus introduced and established in humans as a result of contamination of poliovirus vaccines. It is common in humans, approximately 70% of adults having evidence of infection as measured by antibody to BK. It appears to be the counterpart of SV40 in monkeys, and is probably frequently present in human kidneys. Recent studies by others show a high percentage of renal transplant cases releasing virus after immunosuppression.

The virus has been purified and has the physical properties of SV40. That is, it is about 45nm in diameter, has a density of 1.34 in CsCl, and has the supercoiled, circular DNA which sediments at 21S.

3. JC virus. The JC virus was isolated by Padgett, et al from a case of PML. It grows only in human fetal brain cells, and we have not been able to propagate this virus in sufficient quantity or consistency. It is a difficult virus to study because of its narrow and restricted host range.

In limited studies, we have found that this virus also induces T-antigens immunologically similar to those of SV40, since JC infected cells show a reaction with SV40 T-antibody.

4. Characterization of Hamster Syncytial (Foamy) virus. A recently discovered hamster syncytial or foamy virus was isolated from an SV40 hamster tumor. The major work on this new agent was done by Ms Phyllis Fabisch and Dr. J. Hruska, who have characterized this virus biologically and biochemically. It is a typical foamy virus morphologically, with virions that measure about 100 nm in size; the virus matures by a budding process. Like all foamy viruses, it contains reverse transcriptase. These viruses comprise the second group of "non-oncogenic" viruses with this property.

Preliminary studies indicate that this virus, like the RNA tumor viruses, has a large 60 to 70S genome. If confirmed, this is the first evidence that these viruses have a single large RNA as its genome.

Significance to Biomedical Research and the Program of the Institute

Studies on the relationships in biological as well as biochemical and biophysical properties of RNA- as well as DNA-containing "slow" viruses to the tumor viruses have provided new information which may help to explain mechanisms involved in infection by these viruses. These studies have shown that certain viruses (RNA as well as DNA) which have the potential to "integrate" viral genes in host cells may be important in slow, chronic, or latent infections.

Proposed Course of the Project

Studies will continue on complete biological and biochemical characterization of the human papovavirus. The oncogenic properties of these viruses in vitro as well as in vivo will be examined. Studies will be initiated to study the possible role of these viruses in human disease. A collaborative study has been started with Dr. M. Oldstone (Scripps Clinic & Research Foundation, LaJolla, Calif.) to investigate the possibility that BK virus may be involved in immune complex disease in humans.

Honors and Awards (Dr. Takemoto):

1. Visiting professor in tumor virology, University of Hawaii, June-July, 1972.

2. Participated in symposium on tumor viruses at the American Association of Neurologists, Denver, Colo., October, 1972.

3. Participated in meeting in Biohazards and Cancer Research, Monterey, Calif., Feb. 1973.

4. Appointed to Immunology-Epidemiology Segment, Special Virus Cancer Program, NCI.

Publications:

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LABORATORY OF MICROBIOLOGY


| | | |
|---|---|---|
| Summary | | 1 |
| 61 | The effect of $CO_2$ on growth of microorganisms. | 8 |
| 62 | Biochemical studies on staphylococcal and streptococcal cell walls. | 12 |
| 63A | Occurrence, properties and significance of protein iron-chelators in the biochemistry of the acquisition of iron, and possibly other metals, by microbial and animal cells. | 15 |
| 63B | Studies on the nutrition, physiology, and ultrastructure of Staphylococcus aureus. | 16 |
| 63C | Investigation of Gram-positive cocci. | 20 |
| 64A | Studies with synthetic potential antifungal and antitumor agents. | 24 |
| 64C | Studies on mycoplasmal and plant glycolipids. | 25 |
| 64D | Isolation, separation and characterization of antigenic lipid hapten(s) from streptococci. | 29 |
| 65 | Electron microscopy of bacteria in relating structure and function. | 34 |
| 65A | Studies on the genetics of streptococci. | 42 |
| 65A-1 | Group H streptococcal bacteriophages: effects on genetic systems of transformable streptococci. | 44 |
| 65A-2 | Investigation of Group H streptococcal temperate bacteriophages: fundamental characteristics of phage-host interaction; Fate of cellular DNA during vegetative infections. | 47 |
| 65C | Competence development and genetic transformation in Group H (and Group A) streptococci: early events. | 50 |
| 65C-1 | Competence development and genetic transformation in Group H streptococci: nature and role of autolysins. | 53 |
| 65F | The cell wall constituents found in a stabilized L form. | 56 |
| 65G | Characterization of biophysical and physiological properties of L forms. | 57 |
| 65H-1 | Studies on the fate of Group A streptococci phagocytized by human monocytes and polymorphonuclear leukocytes. | 60 |

| | | |
|---|---|---|
| 77-1-F | Studies on *Aspergillus*. | 61 |
| 77-1-G | Genetic study of pathogenic fungi. | 63 |
| 79-B-1 | Host response to pathogenic fungi in neoplastic disease and immunosuppression. | 67 |
| 79-C-1 | Ecologic and aerobiologic studies of *Histoplasma capsulatum*. | 70 |
| 79-D | Immunochemical studies on polysaccharides from fungi. | 72 |
| 79-E | Yeast flora of birds. | 75 |
| 207 | Basic studies on mycoplasma. | 77 |
| 152 | Studies on antibody production and mechanisms of hypersensitivity. | 82 |

PHS-NIH
Summary Statement
Laboratory of Microbiology, NIAID
July 1, 1972 through June 30, 1973

## Introduction

Although the principle administrative problem is inability to promote all those employees who are due and eligible, LM has continued to operate productively with a restricted budget and essentially the same number of personnel. Herald J. Stone (30 years service), Carl Peacock (32 years service), and C. Winslow Renshawe (33 years service) retired; and were replaced, respectively, by John Owens, Blanche E. Lewis, and James E. Nolke. Mrs. Ann C. Eastwood filled the position of secretary to the Mycology Section in May, 1972. Miss Judith Moir, secretary for the Mycoplasma Section, left to take a position in another Institute, and is expected to be replaced in June. Dr. Unnur Petursdottir (Guest Worker from Iceland) left in August to take a paid position in another Institute. In July, two Fellows initiated studies in Mycology (Dr. Sang Jae Kim, Visiting Fellow from Korea; and Dr. Errol Reiss, NIH Post-Doctoral Fellow from Rutgers); and Dr. Kenneth S. Nugent succeeded Dr. Alan D. Glick as incoming Staff Associate. Miss Minerva J. Lawrence left to take a position in another Institute. In June, Dr. C. Lowell Parsons will complete duty as Staff Associate, and will be succeeded on July 1 by Dr. Gary B. Calandra.

The principal mission of the Laboratory of Microbiology is construed to be the advancement of basic knowledge of prokaryotic microorganisms. Organisms studied include bacteria, fungi, mycoplasma, and bacterial viruses. Areas of research include genetics, metabolism, serology and immunology, taxonomy, ultrastructure, biochemistry, and all interrelations thereof. Particular emphasis is on structure-function relations of such isolatable microorganismal components as cell wall, cell membrane, organelles such as mesosomes, the DNA genetic apparatus; and chemical entities such as structural proteins, enzymes, and other biologically active molecules.

## $CO_2$ Regulates Streptococcal Growth

Streptococcus strain Challis (Group H) fails to grow if $CO_2$ is excluded from the culture. If bicarbonate is present, thus eventually supplying $CO_2$, growth eventually occurs after a lag phase. If $CO_2$ is supplied stepwise in increasing amounts which relate to successive doublings in cell numbers, growth is initiated immediately without lag and continues at maximal rate to the stationary phase. The reaction in which $CO_2$ participates to regulate growth is still unknown, despite exploration of several known cycles involving $CO_2$ fixation. However, the $CO_2$ requirement appears inversely related to levels of a hypothetical and undefined compound which is metabolized during growth but accumulates in stationary phase. Therefore, when the latter is used for inoculum, as is usual, the $CO_2$ requirement for immediate and maximal rate growth is low (0.3%)--but becomes progressively higher (1.8%, 2.4%) as the compound is presumably metabolized during growth. Additional evidence for this is supplied by incubating the inoculum for an hour in a non-growth-supporting buffer; after this "metabolizing" period, the $CO_2$ requirement for immediate and maximal growth is 2.4% instead of 0.3%. The cumulative evidence, which

has also been obtained from bacteria other than streptococci, indicates that $CO_2$ is a general and regulatory requirement for growth; and that the usual lag period is an artifact representing the time required for the inoculum to produce the $CO_2$ concentration needed in the presence of whatever level of hypothetical compound is present in the inoculum. Aside from general importance, the findings have specific applications in relating growth and metabolism to stages in achievement of genetic competence in streptococci. (Repaske)

Mesosomes are Different than Membranes

Continued analysis of membranes and mesosomal vesicles isolated from Staphylococcus aureus 6538P shows more differences. The preponderance of high molecular weight (MW) proteins and 15% more total protein in membranes, was previously shown. Mesosomal vesicles (MV) have more proteins of low MW, and one (MW 35,000) is present in a concentration 4 times greater than in membrane. The protein to lipid ratios are 2:1 in membrane and 1:1 in MV. Nevertheless, there is 9% more total lipid in MV: and even though the same types and numbers of fatty acids are present in each, MV contain 48% more than does membrane. Enzymatic and gas-liquid chromatographic analyses show that 15-20% of dry weight of MV is glycerol, as opposed to 4-6% in membrane. The glycerol is phosphorylated, with a ratio to phosphate of 1:1. This component may be associated with the new neutral lipid fraction (extractable only by butanol-pyridine-acetic acid, as reported last year), or may represent a lipoteichoic acid known to be associated with unspecified membranous fractions of some other gram-positive bacteria. The continuing revelation of differences between membranes and mesosomes, and particularly planned studies for characterization of the unique lipid-glycerol-phosphate component, make it more likely that the debated functional role of the mesosome can be solved. [Theodore, Huff; Collaboratory, Panos (not NIH]

Isolation of M-Protein from "Difficult" Streptococcal Serotypes

A deterrent to possible future polyvalent M-protein "vaccines" to protect humans against Group A streptococcal disease is the poor yield and immunogenicity of M from certain common serotypes. These "difficult" types also produce lipoproteinases or serum opacity factors, which generally are co-purified with M's, have a related but different serotype specificity of their own, and are of unknown significance in human disease or antibody response. Serotype M4 is the most common of these, and this year we initiated studies of its production and yield of M. New approaches included examination of both cells and superantant media; use of concentrated and ultrafiltered media; and incubation with stirring under 5% $CO_2$: 95% $N_2$ during automatic pH regulation. The working approach is to obtain M as little altered as possible--as from media, or as extracted from cells by methods other than the classic mild acid hydrolysis. The type 4 strain used was found to liberate M into the medium; this M was best obtained by ultrafiltration and subsequent elution by NaCl (ionic strength 0.04 to 0.05) at pH 6.0 from a carboxymethyl cellulose (CMC) column. A yield between 0.5 and 1.0 mg per liter resulted; the crude material reacted with type-specific (but not group-specific) antiserum and was sensitive to trypsin inactivation. Of various treatments of cells (NaCl, Triton, Nonidet, SDS, ammonium bicarbonate, etc.), the most successful was freezing, extraction by lithium chloride, and elution with 0.01 M phosphate buffer from a CMC

column. This product included M, but also reacted with group antiserum and normal rabbit serum. These non-specific reactions were eliminated by subsequent elution with a stronger (0.2M) phosphate buffer. The methods demonstrate the feasibility of gentle extraction and initial purification procedures, but require extension to assure good yields, the absence of non-specific components such as lipoproteinase or a "M-associated" protein, and the immunogenicity of the final material. (Schade)

## Relationships of Microbial and Plant Glycolipids Shown

Glycolipids (GL) were extracted from 18 bacteria and 53 higher plants. Some of the bacterial GL were purified on silicic acid columns by a newly-devised method of sequential elution of the original extracts; others resulted from the older methods of water washing and DEAE or Sephadex column chromatography. A new reagent, aniline malonate in ethanol, provided a more sensitive, more easily detectable, odorless method of detecting carbohydrate spots on paper chromatograms. All streptococcal and staphylococcal GL tested possess glucosyl determinants, and, therefore cross-react in complement fixation tests against streptococcal antisera. Some few tested also react with antisera to Mycoplasma pneumoniae, which has both glucosyl and galactosyl determinants. GL from Lactobacillus bifidus and Micrococcus lysodeikticus was shown to have mannosyl units, and consequently did not react with streptococcal antisera. Forty-one plant GL reacted with M. pneumoniae antisera, and analysis of two (carrot and parsnip) showed only galactosyl groups (as previously shown in spinach) as the probable determinants accounting for the M. pnuemoniae cross-reaction. Hybrid membranes were prepared by reaggregation of one streptococcal and two plant purified GL with lipid-depleted membrane protein from Acholeplasma laidlawii. As in previous work with M. pneumoniae GL, the reaggregates were immunogenic in rabbits and produced antisera with specificity for the GL sugar moiety; whereas GL alone, or simple mixtures of GL and protein were unsuccessful. These findings extend the range of experience with glycolipids to which man may be immunologically exposed, and verify the significance and specificity of the carbohydrate determinants. They offer interesting speculation, but no solutions as yet, on the possible participation of widespread GL with common determinants in production and effects of "normal" antibody in humans. (Prescott, Caldes, King; Chanock, Greenbert, Brunner, James - LID)

## Transduction of Group H Streptococci Achieved

Although we discovered and for several years have utilized genetic exchange by phage-mediated transduction in Group A streptococci, no such system has been available for Group H streptococci which include genetically transformable strains. Following last year's discovery of Group H phages, intensive efforts were made to transduce antibiotic resistance markers into Group H streptococci--using both Group A and Group H phages. Following numerous failures to transduce "normal" streptococci with phages induced from antibiotic-resistant lysogenic strains or with phages lytically infecting an antibiotic-resistant strain at various temperatures, success was achieved by the latter method only after nitrosoguanidine (NTG) mutagenization of the prospective recipient streptococci. In this fashion, four NTG-survivor isolates of strain SBE were transduced to streptomycin-resistance by phage ø227

propagated at 37 C in streptomycin-resistant strain Wicky. The nature of the NTG-induced mutation making this possible is as yet unknown, and we are still attempting to transduce other and better characterized strains. Nevertheless, these recent findings make possible a more complete study of genetic exchange in a single group of bacteria in which systems of lysogeny, transformation, transfection, and transduction have all been described. (Colon, Leonard, Parsons, Cole)

Streptococcal (Group H) Bacteriophages and Genetic Transformation

Recently-discovered temperate bacteriophages were shown last year to inhibit genetic transformation when lysogenizing previously transformable Group H streptococci. Most of the few transformants resulting were free ("cured") of prophage; and the lysogenic cells representing the bulk of the culture failed to bind deoxyribonucleic acid (DNA). In these respects, the system differs from a somewhat similar one in lysogeny-inhibited Bacillus subtilis. However, it now appears that a few streptococcal transformants remain lysogenized with prophage which, when induced, can lysogenize and inhibit transformation of previously transformable cultures. The presumption is that an unspecified bacterial mutation has negated the usual prophage effect on transformation in these few lysogenized cells. More interestingly, however, if the total DNA from such mutants is used to transform competent cultures, none of the cells becomes lysogenized, but some produce progeny phage (i. e., transfection). This suggests that the prophage locus is not integrated into the mutant bacterial chromosome, or does not recombine during transformation--and may, therefore, exist as a separately replicating extra-chromosomal unit, or plasmid. (Parsons)

Phage-Host Interactions in Group H Streptococci

Although the occurrence, host range, and some genetic effects of temperate phages from Group H streptococci have been examined in the last two years, no fundamental information on the phage-host system was available. This year, essential studies of vegetative infection of a susceptible indicator strain (WE4, a Wicky derivative) with phage ø227 were initiated. Of 7 methods of phage purification, the most efficient was treatment of phage lysates with diatomaceous earth, followed by filtration and centrifugation. Stable phage stocks with titers of 1 to 4 x $10^{11}$ per ml were obtained. These phage adsorb rapidly (95-99% complete in 7-8 min.) to susceptible cells; the adsorption does not require calcium, and is independent of different concentrations of magnesium, zinc, potassium, and sodium. Since a part of the study concerns the fate of host cell DNA, and requires incorporation of radioactive precursors which cannot be followed in the usual media, a defined medium based on AOAC (Difco) base was developed and shown to be adequate for streptococcal and phage growth, transformation, and incorporation of radioactive thymidine. Latent and rise periods for ø227 in this medium are somewhat longer, and burst sizes smaller, than in undefined medium. In the latter, however, prior heating (presumably inactivating an unknown heat-labile substance) reduces burst size; and citrate inhibits productive infection by inhibiting cell lysis. During phage infection, tritium-labelled host DNA is not degraded to acid-soluble fragments; and the same DNA taken at intervals, give a constant frequency of co-transformation of two linked markers. Also, hydroxyurea (which inhibits

conversion of ribonucleotides to deoxyribonucleotides) prevented phage replication--thus indicating that de novo phage DNA synthesis did not occur in its presence and that no precursors were available from degradation of host DNA. The findings suggest that, in this phage-host system, vegetative infection by phage does not involve or require alteration of the cellular DNA. These studies establish a basis for systematic study of effects of phage infection and lysogeny in Group H streptococci. (Nugent)

Early Events in Streptococcal Competence Defined

Three distinct events occur sequentially during development of genetic competence in Group H streptococci, and each has now been shown to require separate periods, of different durations, of protein synthesis. Production and release of competence factor (CF) requires 30 minutes or less of uninterrupted protein synthesis. Binding of CF, to initiate competence, requires 5 minutes; and those events resulting from binding need another 30 minutes. The pH 2-induced aggregation of cells, formerly thought to represent achievement of competence, now appears related only to cell-bound CF, and does not require later events. Although other studies from this Laboratory have strongly associated the appearance of a new protein and a capacity for autolysis with normal achievement of competence, it was shown that these can be diminished by appropriately-timed trypsin treatment without eliminating subsequent transformability. These observations supply further impetus for studies to separate possible non-essential but indicator events from those which are needed for uptake and recombination of new genetic information. (Leonard, Colon, Cole)

Competence-Associated Autolysis in Group H Streptococci

Streptococci capable of genetic transformation undergo prior phenomena which include appearance of a new protein and susceptibility to a stimulated autolysis. The latter is normally correlated in extent with the frequency of subsequent transformation, although other work in the Laboratory suggests that its extent--or even detectability of the new protein--can be diminished by laboratory manipulations without eliminating transformability. Genetically competent cells release a lysin or other indicator streptococci into the culture medium. Presumably, this lysin represents a released excess of the autolysin which initially acts, in unknown fashion and to unknown extent, on cells during competence achievement. In last year's preliminary studies to characterize the lytic enzyme, it was found to lyse also some--but not all--strains of serogroups A, E, H, and K; and did not attack strains of other serogroups B through S. Extension to 17 strains of Group H--most of which are non-transformable--showed all to be lysed, but in variable degree. Susceptibility was unrelated to transformability, but weakly or non-susceptible strains are those which recent serologic data suggests may not really belong to Group H. Overall, the evidence points to a substrate common to walls of many streptococci, and suggests that factors controlling timing and production of autolysin are of more importance in competence than simply the availability of a susceptible substrate. (Ranhand, Cole)

Distribution of the Two Mating Types of Histoplasma capsulatum

The recent discovery of heterothallism in H. capsulatum made it possible to study the distribution of the two mating types in nature as well as among isolates from human infection. Over 800 soil isolates and 180 human isolates

obtained from 13 endemic states of the U. S. were tested. It was found that the ratio of + and - types among soil isolates was 1:1. Among clinical isolates, however, the - type was always more frequent with the range of 9 (-): 1 (+) to 20 (-) : 0 (+), depending on the geographic area. The results suggest that the - type is either more virulent or gains access to the human body more readily than the + type. (Kwon-Chung)

New Heterothallic Aspergillus Described

Genetic studies of Aspergillus fumigatus (which can cause human disease) have been impossible because of its lack of a sexual state. Six isolates of a new Aspergillus were cultured from the eyes of rabbits. The fungus was found to be the only heterothallic species among the members of the Aspergillus fumigatus group. The asexual fruiting structures,pigment of colony, optimum temperature for growth, and minimum inhibitory concentration of Amphotericin B are the same as for A. fumigatus. There are, however, two mating types among the strains, and these can be used as the biological tool for a genetic study. (Kwon-Chung, Kim)

Serotypes Described in Another Species of Yeast

In only two species of yeasts have distinct antigenic serotypes or groups been demonstrated. Three serotypes have been characterized recently from three reference strains of Saccharomyces telluris and designated as A, B, and C. One reference strain of Torulopsis bovina, the imperfect form of S. telluris, belonged to serotype B. The serotypic differences appear to be due to mannans on the yeast cell surface. One hundred and forty-one strains of S. telluris isolated from four columbid (pigeons and doves) were serotyped. All 98 strains of this yeast isolated from Columba livia, feral pigeon, belonged to serotype B. Three other columbid species, C. leucocephala, white-crowned pigeon; C. fasciata, band-tailed pigeon; and Zenaidura macroura, mourning dove, harboured strains of serotype C only. Serotype A has not been isolated from any of these avian species to date. The ecologic significance of the serotypic relationship to the avian species from which the yeasts were isolated is unclear. Studies are in progress to determine the serologic properties of the three serotypes as compared to those of other yeasts. (Hasenclever)

First Characterization of a Plant Mycoplasma (Spiroplasma)

An agent recovered from citrus "stubborn" disease was studied physiologically and serologically, and so described as a new organism, Spiroplasma citri--the first mycoplasma of plant origin to be characterized. Two isolated from different sources produced mycoplasma-like colonies on solid medium and each possessed biochemical and genetic properties like those of other mycoplasmas. Serum or cholesterol was required for replication, which was optimum at 32 C. The agents contained viable particles passing 220 nm filters, and were resistant to penicillin. Extensive testing versus antisera against 50 known animal or saprophytic mycoplasmas and acholeplasmas failed to show any serologic relationships although there was a reaction with a similar but uncultured organism from corn stunt disease. S. citri grows as motile, helical filaments in liquid media, has no cell wall nor organelles, and is uniquely infected with a tailed bacteriophage. Both it and the corn stunt agent, as well as some

acholeplasmas multiply in leaf hopper vectors of corn stunt and other plant diseases, but a vector relationship in "stubborn" disease is unknown. This new organism provides unique opportunities for studies of membrane structure and function--especially in relation to shape, motility, and phage infection; and experimental infection of insects with this and similar prokaryotes offers new techniques in studies of virulence and pathogenesis. (Tully, Cole; Collaborators, Bové and Whitcomb)

Bacterial Adjuvants and Cellular Immunity in Protective Mechanisms and Immunosuppression

Adoptive immunity transfer techniques in inbred histocompatible guinea pigs have been a major approach to investigation of mechanism involved in the pathogenesis of autoimmune diseases, in immune deviation phenomena, and in the immune protection induced against certain pathogens such as Mycobacterium tuberculosis. By appropriate manipulation of conditions such as time of transfer of lymphoid cells, and of age and genetic background of donors and recipients, certain components only of a given immune response can be transferred. In the autoimmune disease studies, an age, strain, and sex-dependent chronic form of autoimmune encephalomyelitis has been shown to mimic human demyelinating diseases, with evidence of several cycles of demyelination and remyelination. By electron microscopy, multiple bouts of disease and recovery can be traced, indicating that the guinea pig could provide an animal model for multiple sclerosis. (Stone)

Serial No. NIAID 61
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The effect of $CO_2$ on growth of microorganisms

Previous Serial Number: Same

Principal Investigator: Dr. Roy Repaske

Other Investigator: Mrs. Anne C. Repaske, Guest Worker

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 24/12 | - | 6/12 | 30/12 |
| Professional | 12/12 | - | 6/12 | 18/12 |
| Other | 12/12 | - | - | 12/12 |

Project Description:

Objectives:

Analyze the effect of $CO_2$ on growth of selected heterotrophic bacteria; i.e., E. coli (aerobic, simple inorganic medium) and a Group H Streptococcus (anaerobic, complex organic medium); to determine the physiological response of the organism. Attempt to identify the site at which $CO_2$ acts to initiate cell growth.

Methods Employed:

Cells were grown in a 1 liter fermentor under a controlled gas environment. The flow rate of each gas ($N_2$, $O_2$, $CO_2$) could be regulated independently to permit rapid and accurate changes in the composition of the gas mixture. Growth was monitored and recorded continuously by a spectrometer through which the culture medium was constantly cycled. Analyses of substrate utilization and fermentation products were made with standard chemical and enzymatic tests.

Major Findings:

When $CO_2$ is excluded from the environment, Streptococcus (Challis) and E. coli fail to grow in media which otherwise supports heavy cell growth. Carbon dioxide is an essential growth nutrient which is not generally recognized even though there were reports by others who demonstrated the qualitative requirement for $CO_2$ for cell growth. Since $CO_2$ is not specifically supplied to growing bacterial cultures, it is incumbent on the organism to produce the carbon dioxide necessary to initiate its own growth. We have found that the time required to produce this carbon dioxide metabolically is probably related to the normal lag period in cultures. Streptococcus (Challis), which has a relatively high $CO_2$ requirement (2.4%), has a long lag period; whereas E. coli, which has a low $CO_2$ requirement (0.075%), has a short lag period. When $CO_2$ is exogenously supplied and its concentration in controlled, both organisms began growing immediately without lag periods. If the $CO_2$ supply was subsequently stopped at any time, growth abruptly stopped until $CO_2$ was again provided. The rate of cell growth could also be regulated by supplying a sub-optimal concentration of $CO_2$. These data demonstrate that the lag period can be extended indefinitely or eliminated completely at will by $CO_2$ control. The lag period simply reflects a nutritional deficiency and can not be considered a "normal" physiological state of bacterial culture growth.

Streptococcus cultures inoculated with cells from a stationary phase culture grow immediately at maximal rates if 0.3% $CO_2$ is supplied. After about one cell doubling, the $CO_2$ must be increased to 1.8% and after an additional 50% increase in cell numbers the $CO_2$ must again be increased to 2.4%. Thereafter, the $CO_2$ optimum remains at 2.4%. The increasing $CO_2$ requirement seems related to changes in a cellular compound originally present in the inoculum; the change could occur either by metabolism or by dilution during cell division. Neither exhaustive washing of the inoculum nor preincubation of the inoculum for 1 hr at 0 C altered the requirement for successive increases in $CO_2$. If, however, the inoculum was preincubated in buffer for 1 hr at 37 C during which time no growth occured, the inoculum changed and now required 2.4% $CO_2$ for immediate maximal growth rates. The compound which was metabolized and spared the high $CO_2$ requirement has not yet been identified. E. coli differed from Streptococcus and has a uniform $CO_2$ requirement regardless of the physiological age of the inoculum.

Carbon dioxide is known to be fixed by $C_3$ compounds to produce $C_4$ dicarboxylic acids needed for biosynthesis of aspartic and glutamic acids. The general assumption has been that these reactions explain the need for $CO_2$. Streptococcus and E. coli grown on media supplemented with aspartic and glutamic acids still require $CO_2$. In another test, E. coli was grown on acetate as the sole source of carbon. Under these conditions the cells synthesize $C_4$ dicarboxylic acids immediately and $CO_2$ should not be needed. Carbon dioxide was still required. Aspartate, glutamate, arginine, purine, and pyrimidine bases are primary recipients of fixed $CO_2$. Addition of all of these compounds did not change the $CO_2$ requirement. Reactions involving the fixation of $CO_2$ into these compounds are undoubtedly important, but adding the products of these reactions did not spare the $CO_2$ requirement. It is, therefore, concluded that $CO_2$ functions in some other unknown reaction in its growth regulating function.

Significance to Bio-Medical Research and the Program of the Institute:

The detailed quantitative data obtained with two divergent physiological types of microorganisms and the qualitative results reported by others on over 100 species of microorganisms indicate $CO_2$ is a general requirement for growth of microorganisms. Both E. coli, representing a nutritionally simple aerobic heterotroph and anaerobically grown Streptococcus representing a nutritionally complex heterotroph require $CO_2$ for initiation of growth as well as maintenance growth after growth has become established. The "normal" lag period which precedes culture growth results from a deficiency of $CO_2$ and represents the time required by the inoculum to produce metabolically the requisite $CO_2$ concentration. The lag period, previously considered a stage of growth, can now be viewed as an artifically induced stage which is eliminated by providing the proper $CO_2$ concentration. Old empirical procedures employed to minimize the lag period can be understood in terms of the $CO_2$ requirement. Large inocula shorten the lag phase because a larger concentration of cells produces $CO_2$ more rapidly. The use of exponential phase cells as inocula provides metabolically active cells which produce $CO_2$ more quickly. The initiation of growth in shaken cultures is promoted by not agitating them at the beginning because agitation promotes diffusion of $CO_2$ from the medium.

This effect of $CO_2$ on cell growth is of fundamental importance because it is a general phenomenon and directly controls cell growth. The effect of $CO_2$ cannot be explained by fixation of $CO_2$ into essential compounds because organisms grown in media supplemented with products of these reactions still require $CO_2$.

Practical applications of these results would result whenever it is advantageous to minimize the time to grow cells. In the production of biologicals, growth time could be shortened; in clinical laboratories, earlier diagnosis and treatment of microbial diseases should be possible because pathogens could be identified sooner, and antibiotic sensitivity tests could be performed in a shorter time.

Proposed Course of Project:

Several methods will be used to determine the specific metabolic system in which $CO_2$ participation controls cell growth. Various microorganisms including mutants will be used to localize the $CO_2$ target. Each organism will provide a unique test system differing from the others by some characteristically different physiological or metabolic property. These results should provide leads to identify the general metabolic system (glycolysis, Krebs Cycle, phosphorylation, protein synthesis, etc.) in which $CO_2$ has a critical function. The specific system can then be segregated to identify the particular reaction(s) in which $CO_2$ participates. Tracer studies will be used where applicable to confirm and identify the fixed $CO_2$.

Honors and Awards:

Invited Seminar, Georgetown University, March 9, 1973, "$CO_2$ Requirement for Cell Growth."

Lecturer, Foundation for Advanced Education in the Sciences, inc.:
a) organized and presented course in "Bacterial Metabolism," fall, 1972
b) organized "Advanced Topics in Microbiology," spring, 1973.

Publications:

None

Serial No. NIAID 62
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biochemical Studies on Staphylococcal and Streptococcal Cell Walls

Previous Serial Number: Same

Principal Investigator: Dr. Eskin Huff

Other Investigators: None

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 24/12 | - | - | 24/12 |
| Professional | 12/12 | - | - | 12/12 |
| Other | 12/12 | - | - | 12/12 |

Project Description:

Objectives:

To apply recently developed methods for the quantitative analysis of polyols, sugars, hexosamines, and amino acids to hydrolysates of Streptococcal and Staphylococcal cell walls, membranes and mesosomes.

To continue development of methods for the determination of the acidic, basic, and neutral fractions on gas liquid chromatography.

Methods Employed:

Methods for isolation of cell walls, growth of cells, hydrolysis of dry material, separation of neutral, acidic, and basic material on ion exchange columns and by gas liquid chromatography were described in previous reports. A method for the gas liquid chromatography of hexosamines as their butane boronates (using 3% OV-17 on Chrom Q) was obtained from Dr. Frank Eisenberg (NIAMDD).

A modification of the procedure for sugars and polyols was necessary in order to get reproducible results with glycerol. Samples were mixed with internal standards of mannitol and 2-hydroxy, 2-hydroxymethyl-1, 3-propanediol (HMMPD). All drying steps during derivitization were carried out at 37° and stopped as soon as samples were dry. On occasion the mannitol to HMMPD ratio showed a loss of HMMPD. In these cases the data was discarded since the glycerol would also show a loss.

Major Findings:

Methods were developed for the determination of polyols, sugars, hexosamines, and amino acids using gas liquid chromatography and were reported previously. Time studies of the hydrolytic release of each of these compounds from Streptococcal and Staphylococcal materials were run to establish optimum conditions for hydrolysis. Using 2 N HCl at 100° C in a sealed tube under argon for hydrolysis of samples, a study was made of the release of glycerol, ribitol and anhydroribitol, rhamnose, galactose, and glucose from cell walls, membranes and mesosomes obtained from these organisms. Time for maximal release of glycerol and anhydroribitol was four days. Maximal release of glucose, rhamnose and ribitol was one hour.

Using these methods comparative studies have been made of the walls, membranes and mesosomes of *Staphylococcus aureus* 6538P (prepared by Dr. Theodore). It can be seen in the table below that all three structures had distinct and different analyses.

PERCENT OF DRY WEIGHT

| | CELL WALLS | MESOSOMES | MEMBRANES |
|---|---|---|---|
| Glycerol+ | 1.1 | 15 → 20 | 5.4 → 5.6 |
| Anh. ribitol+ | 4.8 | 0.36 → 0.40 | 0.34 → 0.47 |
| Ribitol* | 2.0 | 0.43 | 0.32 |
| Glucose* | 0.14 | 0.91 | 0.90 |

\+ 4-day hydrolysis
* 1-hour hydrolysis

The glycerol content of membranes and mesosomes has been verified using glycerol kinase and a coupled enzyme system.

A phosphate analysis showed equal amounts of glycerol and phosphate in membranes and mesosomes (phosphate to glycerol ratio: 0.98 in membranes; 1.13 in mesosomes). Phosphatase treatment of a partial hydrolysate of mesosomes and membranes also showed equimolar release of glycerol and inorganic phosphate. Much of the glycerol (75% in mesosomes and 50% in membranes) is released very slowly

Serial No. NIAID 63B
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the nutrition, physiology, and ultrastructure of Staphylococcus aureus

Previous Serial Number: Same

Principal Investigator: Dr. Theodore S. Theodore

Other Investigators: Dr. Eskin Huff
Dr. Kenneth Nugent

Cooperating Units: Dr. Eugene Weinbach
Laboratory of Parasitic Diseases, NIAID (Serial No. 123-C)

Dr. Charles Panos
Dept. of Microbiology
Jefferson Medical College
Philadelphia, Pennsylvania

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 24/12 | - | - | 24/12 |
| Professional | 12/12 | - | - | 12/12 |
| Other | 12/12 | - | - | 12/12 |

Project Description:

Objectives:

To isolate and characterize by cytological and biochemical methods the membrane system of Staphylococcus aureus; more specifically, to examine the distribution of certain enzymes and compounds in the membrane system in order to allow for the biochemical, physiological, and histological differentiation of mesosomes and plasma membrane.

Methods Employed:

Routine bacterial and cultural procedures; spectrophotometry, polarography, paper and thin layer chromatography, gas-liquid chromatography, polyacrylamide

gel electrophoresis and routine metabolic, enzymatic and radioisotope procedures; differential and density gradient centrifugation techniques and electron microscopy.

Major Findings:

1. Chemical composition of plasma membranes and mesosomes of Staphylococcus aureus. Although mesosomal and plasma membranes consist mostly of protein and lipid (75-80%), the relationship of protein to lipid between the two membrane fractions is markedly different. The ratio of mesosomal protein to lipid is 1:1 while that of membranes is 2:1. These findings strongly suggest that specific quantitative chemical differences exist between the two membrane fractions. We have already reported that membranes contain a higher concentration of high molecular weight proteins while the mesosomes have a higher concentration of low molecular weight proteins. On sodium dodecyl sulfate containing polyacrylamide gels, we find one prominent protein band of approximately 35,000 molecular weight whose concentration is 4 times greater than that found in the corresponding membrane fraction. Similar studies on their fatty acid composition have shown that the same types and numbers of fatty acids are present in both membrane fractions; however, the concentration of fatty acids is 48% greater in the mesosomes. Chloroform-methanol extraction and thin layer chromatographic analysis of the lipids showed no qualitative or quantitative differences; but butanol-pyridine acetic acid extraction revealed what appears to be a new lipid component which is present almost exclusively in the mesosomal membranes. In pursuing this lipid, we analyzed the membrane fractions by gas-liquid chromatography following extended acid hydrolysis in 2 N HCl and found that 15-20% of the dry weight of mesosomes consisted of glycerol while the membranes contained only 4-6% (ratio of mesosomal to membrane glycerol is 4:1). Glucose, ribitol and anhydroribitol were also present, but each to an extent of 1% or less. Similarly analyzed cells contained only 1% glycerol, indicating that the membrane fractions were not contaminated by wall material (S. aureus ATCC 6538P cell walls contain a ribitol rather glycerol teichoic acid). The above glycerol data was verified by enzymatic analysis following neutralization of the acid hydrolysate. Phosphate analysis on the same membrane fractions showed that a 1:1 ration of glycerol to phosphate existed. Treatment of this glycerol-phosphate complex with alkaline phosphatase resulted in the release of inorganic phosphate and glycerol; indicating that the glycerol is present in an esterified form. At present, the exact structure or function of this compound is not known. We believe that either it is associated with a specific neutral lipid fraction extractable only by butanol-pyridine-acetic acid treatment or it is a lipid-teichoic acid compound (polyglycerolphosphate) which has up to now been found only in the plasma membranes of Gram positive bacteria. (Theodore, Huff, and Parsons) (See also Project NIAID 62, in part)

2. Enzymatic activities associated iwth mesosomal and plasma membranes. Previously, we reported that the cytochrome activity is primarily localized in the plasma membrane fraction. The concentrations of cytochromes A, B, and cytochrome oxidase O are 3-4 times greater in membranes than in mesosomes. These measurements pertain to the terminal sequences of the electron transport

chain. In collaboration with Dr. E. Weinbach, the characterization of the complete electron transport system in S. aureus has been determined by oxygen electrode polarographic techniques. Succinate, malate, lactate, alpha-glycerol phosphate and NADH oxidases are most active in the plasma membrane fraction. The mesosomes showed only marginal oxidase activities. Currently, studies on the individual dehydrogenases are being done to determine if any increased levels of specific respiratory enzymes are associated with the mesosomes.

It has been reported that mesosomes of Micrococcus lysodeikticus isolated by lysozyme treatment contain extremely high levels of an autolytic enzyme which is active against the parent organism (Owen and Freer, 1972, Biochem. J. 129, 907). Using similarly designed experiments and a variety of physiological conditions, we were unable to find any autolytic activity associated either with mesosomes or membranes of S. aureus. In all probability, the above activity of M. lysodeikticus mesosomes is due to the adherence of lysozyme to the membranes. Staphylococcal mesosomes prepared in the presence of lysozyme (lysozyme does not attack staphylococcal cells) were also capable of autolyzing M. lysodeikticus. The presence of other degradative enzymes, presumably associated with the mesosomal membranes will also be analyzed (i.e., acid and alkaline phosphatases, ATPase and penicillinase). (Theodore and Weinbach)

3. Characterization of ribosomal proteins in S. aureus. Bacterial ribosomes are found in association with the plasma membrane and in the cytoplasm. Under the appropriate experimental conditions, both types of ribosomes are capable of in vitro polypeptide synthesis. However, the physiological role of membrane bound ribosomes, as well as the nature of their attachment to the membrane is obscure. It has been suggested that the two sets of ribosomes are structurally and functionally different. To test this, we have analyzed the proteins of cytoplasmic and membrane ribosomes using polyacrylamide gel electrophoresis. Protoplasts were lysed in hypotonic buffer and collected by differential centrifugation. Membrane bound ribosomes were dissociated from the membrane by sodium desoxycholate treatment and also collected by differential centrifugation. The RNA portion of the ribosomes was removed by glacial acetic acid treatment and the resultant proteins analyzed on sodium dodecyl sulfate polyacrylamide gels. The gel patterns of the cytoplasmic ribosomes are both qualitatively and quantitatively distinct from the membrane ribosomes. Cytoplasmic ribosomes have 19 distinct protein bands whereas membrane ribosomes have only 15. Also, these patterns resemble neither the mesosomal nor plasma membrane protein patterns indicating that the ribosomal proteins were not contaminated by any membrane fragments during preparation. What relationships these protein differences have pertaining to the functional activities of ribosomes (specificity of polypeptide synthesis) or the attachment sites to the membrane is not known. (Nugent and Theodore)

Significance to Bio-Medical Research and the Program of the Institute:

Morphologically, the most distinct feature of the bacterial membrane system is the mesosome. The structure of this unique organelle, which arises

by an invagination of the plasma membrane, has been described in great detail in a number of bacteria. However, little is known about its functional role in the cell. Our approach to this problem has been to characterize the chemical composition of this organelle, and attempt to correlate these findings to its possible function. Using the chemical composition of plasma membranes as a basis for comparison, we have shown increased quantitative differences of protein, fatty acid, and lipid composition in the mesosomes. The lipid composition is extremely significant since we find that 15-20% of the dry weight of the mesosome is composed of glycerol while only 4-6% glycerol is present in the corresponding membranes. Also, there is a 1:1 ratio of glycerol to phosphate. These findings suggest that this glycerol-phosphate complex is either associated with some as yet undescribed neutral lipid fraction or is the lipid-teichoic acid (polyglycerol phosphate) component previously found only in the plasma membranes of Gram-positive bacteria. If the latter is true, then the mesosome would appear to function either as the site of lipid-teichoic acid biosynthesis or as a storage center for this compound. The function of the membrane teichoic acid is to regulate the movement of cations between the exterior and interior environments of the cell. Using specific fractionation techniques, we hope to isolate this glycerol-phosphate component intact and characterize its structure. These studies will enable to ascertain what function resides in the mesosome and how it relates to the cell's economy.

Proposed Course of Project:

The following lines of investigation will be pursued:

1. Qualitative and quantitative characterization of mesosomal lipids extracted with butanol-pyridine-acetic acid and isolation and chemical characterization of the glycerol-phosphate component found in the mesosomes.

2. Analysis of specific dehydrogenases associated with respiratory activity and analysis of degradative enzymes associated with the mesosomal membranes.

3. Labelled DNA binding experiments to determine whether or not DNA binds specifically to the mesosomal or plasma membrane fractions.

4. In vitro polypeptide synthesis experiments to determine whether any functional specificities are associated with cytoplasmic and membrane bound ribosomes.

Honors and Awards:

Council representative of NIAID to Interassembly of Scientists Council.

Publications:

None

Serial No. NIAID 63C
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Investigation of Gram-Positive Cocci

Previous Serial Number: Same

Principal Investigator: Dr. Arthur L. Schade

Other Investigators: None

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 26/12 | - | - | 26/12 |
| Professional | 12/12 | - | - | 12/12 |
| Other | 14/12 | - | - | 14/12 |

Project Description:

Objectives:

To effect the isolation, purification, and characterization of the type-specific M protein antigen from the difficultly typable strain of Streptococcus pyogenes, type 4, whose frequency of identification as the etiologic agent in cases of human streptococcal disease is relatively and significantly high.

Methods Employed:

Standard methods of bacteriology, metabolism, and biochemistry. Special methods, as required, include a variety of immunological procedures; radioactivity techniques; spectrophotometry; electrophoresis; chromatography; and ultracentrifugal gradient separations.

Major Findings:

Most efforts have been directed towards the establishment of procedures for the production and subsequent isolation of streptococcal type 4 M protein

from both bacterial culture media and cells, under conditions favoring the maintenance of its native state. Following preliminary trials, we chose the following medium and growth conditions as providing suitable, if restricted, yields of M protein in the culture medium:

(a) 2 X concentrated Todd Hewitt broth fortified with glucose to give a final concentration of 0.055-M and with cysteine to 0.0014-M;

(b) inoculation of 6 liters of such medium with 300 ml of a growing culture of the CDC #0107 type 4 Streptococcus pyogenes, a producer of the serum opacity factor as determined by us;

(c) incubation of the inoculated medium at 37 C with continuous magnetic stirring under a gas atmosphere of 5% $CO_2$ + 95% $N_2$ and at a pH held between 7.3 and 7.45 by automatically-regulated alkali addition.

To eliminate medium constituents that are unable to pass the dialysis membrane under a vacuum of 70 cm Hg, the Todd Hewitt broth was initially ultrafiltrated in the cold at 4 to 8 X the usual concentration. Following various culture incubation times, the cells were removed by centrifugation and the supernatant, in presence of toluene, was again ultrafiltrated through a dialysis membrane. The residue now left in the membrane sausage is the source of crude M protein. Cells accumulated by centrifugation are available for M extraction studies.

Ammonium sulfate precipitation of the M protein from the growth media at 60 and higher percentages saturation was attempted. Despite satisfactory precipitation of M protein, other products were obtained, often colored, which proved difficult later to separate. Use of merthiolate 1/10,000 to effect supernatant sterilization was employed but later judged a possible source of difficulty in further product purification. Sodium azide was used and may prove ultimately satisfactory although it and merthiolate have spectral absorptions in the UV range which make spectrophotometric protein assay inconvenient during isolation procedures.

Initial purifications of ultrafiltrated crude M protein products were effected by gel filtration and/or ionic exchange procedures on anionic and cationic solid media. $D_{\lambda 280nm}$-absorbing fractions, with an estimated molecular weight of 60 - 80,000 and reacting positively with type 4 specific antiserum, were obtained. All such products contained some Group A carbohydrate and require further purification. We attempted to separate the indicated carbohydrate moiety from the M protein-containing product by treating it with 8 molar urea at pH 6.0 followed by salt precipitation. Both specific protein and carbohydrate reactivities remained with the urea-treated material.

The most promising preparation from the concentrated ultrafiltrated growth medium to date has come from a carboxymethyl cellulose column fractionation at pH 6.0 with elution by NaCl at an ionic strength of 0.04 - 0.05. While the preparation was strongly positive with the type specific M antiserum, it was only dubiously positive with the Group A antiserum. The yield of crude product was estimated at 0.5 to 1.0 mg per liter supernatant. Treatment of the

isolated material according to the classical M protein extraction procedure of Lancefield, namely pH 2.0 with HCl and 10 minutes at 95-100 C, yielded a product reacting positively with the type 4 specific antiserum. Trypsin digestion of the same material destroyed this reactivity.

Demonstration that M protein activity is present in culture supernatants whose pH has been maintained at 7.3 to 7.5 raises the expectation that M protein should be extractable from the cells under mild conditions. Samples of washed cells from a 6-hour culture, resuspended in a 0.05-M phosphate buffer at pH 7.4, were extracted for 1 hr at 37 C in molar NaCl; in Triton detergent at 0.5 and 5% concentrations; in Nonidet P-40 detergent at 0.5 and 5% concentrations; and in ammonium bicarbonate at 0.1 and 1.0 molar concentrations. No M protein was found in any extract. Washed cells from another 6-hour culture were extracted for 3-1/2 hours at 35 C in molar LiCl plus 0.1% sodium dodecyl sulfate at pH 7.86 of 0.1-M tris-HCl buffer. No M protein was found in the extract. Cells of the same culture were treated with molar LiCl in 0.1-M acetate buffer at pH's of 3.2, 4.0, and 5.4 (final pH's lay between 4.4 and 6.05). These extracts gave equivocal results. When cells of a subsequent culture were frozen for 96 hours at -10 C and then extracted with molar LiCl in 0.139-M acetate buffer at pH 4.5 for 2 hours at 35-41 C, an eluate containing M protein was obtained. The extracted product was put on carboxymethyl cellulose at pH 6.0 and eluted with 0.01-M phosphate buffer. The eluate was reactive with both normal rabbit serum (NRS) and type 4 specific antiserum (SA). Subsequent elution with 0.2-M phosphate buffer at pH 6.0 gave a product non-reactive with NRS, positive with SA but negative with carbohydra Group A specific antiserum. Further investigations are required to establish the definitive conditions leading to successful repetition of these results.

Significance to Bio-Medical Research and the Program of the Institute:

Currently there are known at least 60 specific types of Streptococcus pyogenes, each characterized by possessing an immunogenically distinguishable surface layer protein, its M protein. As McCarty has observed, not only are the M proteins necessary for the virulence of these organisms "but they serve as the determinant of the prevalent pattern of human streptococcal disease." Lacking adequate knowledge of the structure of these proteins and having "none on the nature of that common feature of this serologically diverse family of antigens that confers the property of resistance to phagocytosis," we shall advance bio-medical research by realizing the objectives of this project. Since type 4 Group A streptococci are frequently identified as etiologic agents in cases of human disease, the successful purification and characterization of its M protein would allow its inclusion in a polyvalent vaccine and enhance its prospective efficacy. Such a result will help to fulfill the program of the Institute, and its interest in streptococcal disease as a special emphasis area.

Proposed Course of Project:

We propose to determine the conditions necessary to assure a good yield of M protein from the type 4, Group A streptococci by means of LiCl extraction,

and from their culture media. With sufficient, partially purified M protein on hand we shall apply suitable immunological, chromatographic, ionic exchange, and electrophoretic techniques including isoelectric focusing in polyacrylamide gels to achieve the purification and physical characterization of this protein. Chemical analyses will follow successful preparation of a pure type 4 M protein. Concomitant with these studies, investigations into the biological properties of the successively more defined M protein products must be carried out. Such investigations include demonstration of the presence or absence of a lipoproteinase activity of a so-called opacity factor of type 4 and other difficultly typable Group A streptococci; the application of a complement fixation test to determine the presence or absence of a non-type specific "M-associated protein" found in purified products from M positive but not M negative strains; bactericidal tests, both direct and indirect, to determine the effectiveness of the isolated M protein to neutralize antiserum in its effect on the survival of streptococci in normal human blood; the immunogenic effectiveness of the M protein product to produce specific antibodies; and, if possible, the demonstration of developed in vivo immunity in mice against homologous type 4 streptococci through use of the purified M protein as antigen.

Honors and Awards:

Member Scientific Advisory Committee, Annual Colloquium, Protides of the Biological Fluids, Brugge, Belgium.

Publications:

None

Serial No. NIAID 64A
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies with synthetic potential antifungal and antitumor agents

Previous Serial Number: Same

Principal Investigator: Dr. Benjamin Prescott

Other Investigators: George Caldes

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | - | - | - | - |
| Professional | - | - | - | - |
| Other | - | - | - | - |

Project Description:

See 1972 Annual Report.

Project has been terminated.

Serial No. NIAID 64C
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on mycoplasmal and plant glycolipids

Previous Serial Number: Same

Principal Investigator: Dr. Benjamin Prescott

Other Investigator: George Caldes

Cooperating Units: Laboratory of Infectious Diseases (NIAID 70C)
Dr. Robert M. Chanock
Dr. Harry Greenberg
Dr. Helmut Brunner
Walter James

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 12/12 | - | - | 12/12 |
| Professional | 6/12 | - | - | 6/12 |
| Other | 6/12 | - | - | 6/12 |

Project Description:

Objectives:

Further production and purification of serologically active glycolipid hapten from Mycoplasma pneumoniae for the preparation of hybrid reaggregates with Acholeplasma laidlawii lipid depleted membrane proteins; and isolation of serologically reactive glycolipids from certain plants and bacteria which cross-react in serological tests with rabbit and human antiserum to M. pneumoniae.

Methods Employed:

The methods used for the isolation and purification of the serologically reactive mycoplasmal and plant glycolipids have been described in previous reports (64C, 1969-70).

Extraction of lipids. The procedures for extracting the antigenic lipid constituents from plant material were adaptations of chemical and physico-chemical procedures described previously (64C, 1969-70). The carbohydrate-rich (glycolipid) fraction was present in the chloroform-methanol 7:3 eluate fraction similar to that of bacterial glycolipids. Final purification of the serologically reactive fraction was carried out by combined subfractionation on columns of silicic acid and DEAE-cellulose. The amount of glycolipid extracted varies greatly from one plant species to another, depending on the method of isolation.

Thin layer chromatography. The CM 7:3 glycolipid fraction was finally chromatographed by two dimensional chromatography on silica gel borate impregnated plates in chlorform-methanol-water (65:25:4 v/v) as described in Project 64D, plates dried and spots detected with periodate-Schiff reagent. The material now showed one spot on the TLC plates. This one spot material was then eluted from several plates for use in the analytical procedures.

Analytical procedures. Carbohydrate, glycerol glycoside, and glycerol were determined by procedures used in previous studies. Hydrolysis of the glycolipids was carried out in extraction flasks for separating water-soluble and hexane-soluble products. The fatty acids (hexane soluble) were weighed for conversion to methyl esters. To obtain the glycerol glycoside, 5 mg of the glycolipid were saponified in 5 ml 0.3 N methanolic sodium hydroxide under reflux for 1 hour, instead of the tetraethylammonium hydroxide used previously. The mixture was shaken with Dowex-50 (H+) to remove sodium ion, centrifuged free of resin and extracted with hexane to remove fatty acids. The water-soluble glycosyl glycerol was concentrated for carbohydrate analysis.

Reaggregation. Carrot and parsnip glycolipids were used for the reaggregation studies. They were selected because of high levels of complement-fixation antigen activity in the CM 7:3 extracts and because of ease of large scale purification. Purified glycolipids by themselves are poor immunogens. In order to render these glycolipids more immunogenic, Razin's procedure (see previous report 64C, 1970-71) for reaggregation was employed in which the glycolipids were mixed with A. laidlawii membrane protein molecules to produce a "hybrid" membrane reaggregate.

Immunization of rabbits. The schedule for the immunization of rabbits with the "hybrid" reaggregates was similar to that employed with the antigens in the streptococcal studies (Project 64D) except that the rabbits received the immunizing doses every two weeks instead of three and were bled three and four and one-half weeks after the first immunizing dose and the sera tested for complement-fixing, metabolism inhibiting and mycoplasmacidal antibody by standard procedures.

Major Findings:

The chloroform-methanol (7:3 v/v) fractions from 53 plants were evaluated for their capacity to fix complement with antibodies to M. pneumoniae or its glycolipids. Forty-one of the plant glycolipids were found to have serologic reactivity with rabbit and human antisera to M. pneumoniae. Each also reacte

with rabbit anti-M. pneumoniae glycolipid serum. Glycolipids from eighteen gram-positive bacteria or L forms (streptococci and Staphylococcus aureus) were also studied and three fixed complement with the M. pneumoniae antisera. The positive reactors included S. aureus E8507, S. aureus ATCC-L 23832 and the L form of Streptococcus pyogenes 64x392, type I. These findings show that certain bacteria and M. pneumoniae share similar lipid antigens. Since carrots and parsnips were two of the vegetables which exhibited a high level of antigenic activity and were available in large quantities, they were studied in greater detail. Purified glycolipids from these two vegetables as well as the "hybrid" membrane reaggregates containing these glycolipids exhibited a high degree of complement-fixing activity. It has previously been shown that human mycoplasmacidal antibody was directed against the glycolipid determinants of M. pneumoniae. Further, specific M. pneumoniae glycolipid antibody was observed to be highly effective in complement-mediated lysis of the organisms. The reaggregated plant glycolipids were found to be efficient in blocking mycoplasmacidal antibody contained in human convalescent serum and in rabbit antiserum prepared against the glycolipids of M. pneumoniae. As expected glycolipid membrane reaggregates were more immunogenic than the glycolipid suspensions alone.

The water soluble glycosides of carrots and parsnips co-chromatographed with a pure commercial product from spinach. Hydrolysis of the isolated deacylation product by acid and enzymatic (galactostat-β-galactosidase) yielded glycerol, and galactose as the only carbohydrate. Furthermore, elemental analysis showed the purified glycolipids to be a digalactosyl diglyceride, molar ratio of carbohydrate-ester-glycerol, 2:2:1. Preliminary analysis of the fatty acid components indicated a predominance of unsaturated fatty acids, largely linolenic. In addition to the analytical studies, it is reasonable to predict that the active plant glycolipids are similar in structure to their M. pneumoniae counterparts based upon immunological reactivity.

Significance to Bio-Medical Research and the Program of the Institute:

In previous studies on the antigenicity of Mycoplasma pneumoniae, it has been shown that M. pneumoniae contains galactolipids as major antigenic components. It has also been shown that spinach contains a digalactosyl-diglyceride that cross-reacts with M. pneumoniae in the complement-fixation test. Thus, the observation that vegetable glycolipids, either in a suspension of a auxiliary lipid such as lecithin or as part of a "hybrid" membrane reaggregate, can stimulate antibodies, which react with M. pneumoniae glycolipids and which kill the organism, suggests that vegetable glycolipids could be responsible for some of the antibody in normal human serum which reacts with M. pneumoniae or its glycolipids. Reaggregated plant glycolipids might be effective in stimulating local defense mechanisms in the respiratory tract and prevent the disease in man caused by M. pneumoniae. Thus, these preliminary studies might be a starting point for a study to learn the effect on the immunological system of various glycolipids derived from natural food plants, as related to bacterial infections.

Proposed Course of Project:

The reaggregated plant glycolipids will be tested for immunogenicity in hamsters using dose responses by intranasal and intraperitoneal routes. New combinations of bacterial membrane proteins and serologically reactive glycolipids from various sources will be investigated both in vitro and in vivo for their antigenic activity and cross-reactivity with M. pneumoniae. Studies are also being conducted on the role of lipids in the antigenic structure of M. neurolyticum and M. fermentans and the cross-reactivity of these lipids with M. pneumoniae. Work on the separation and purification of some of these glycolipids is in progress.

Honors and Awards:

Invited to attend and participate in Workshop on New Approaches for Inducing Natural Immunity to Pyogenic Organisms, Winter Park, Florida, March 21-23, 1973.

Consultant to NIH Library on purchases of certain chemical journals.

Consultant to Vaccine Development Committee, Infectious Diseases Branch, Collaborative Research Division.

Publications:

Barden, J. A., and Prescott, B.: Chemical and serologic properties of Mycoplasma hyorhinis fractions. Infection and Immunity (in press)

Greenberg, H., Prescott, B. Brunner, H. James, W., and Chanock, R. M.: Sharing of glycolipid antigenic determinants by Mycoplasma pneumoniae. Vegetables and certain bacteria. Workshop on New Approaches for Inducing Natural Immunity to Pyogenic Organisms. To be published as a monograph under title of conference.

Serial No. NIAID 64D
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Isolation, separation and characterization of antigenic lipid hapten(s) from streptococci

Previous Serial Number: Same

Principal Investigator: Dr. Benjamin Prescott

Other Investigators: Dr. James R. King
Mr. George Caldes

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 28/12 | - | - | 28/12 |
| Professional | 16/12 | - | - | 16/12 |
| Other | 12/12 | - | - | 12/12 |

Project Description:

Objectives:

The isolation, purification, and characterization of serologically active glycolipids from several groups of streptococci [(A through H K, L, R]; and a continuation of studies on cross-reactive or shared determinants in the streptococcal and other organisms studied.

Methods Employed:

In previous reports we have shown that glycolipids isolated from several streptococcal groups possessed high serological activity in the complement-fixation test. We have now isolated and tested the glycolipids from Groups A through H, K, L, and R streptococci for complement-fixation activity in sera of rabbits immunized with whole cell vaccine. In addition, we have also isolated the glycolipids from several strains of Staphylococcus aureus E8507, ATCC 23832-L form, ATCC 6538P and from Lactobacillus bifidus ATCC 11147, and

Micrococcus lysodeikticus (Schwarz-Mann X3056). In our earlier studies on the separation of lipid fractions from streptococci, we utilized combined column chromatography on Sephadex G-25 fine, with DEAE-cellulose, and subfractionation on columns of silicic acid. The glycolipids still contained small amounts of impurities (phospholipids) requiring further column chromatography and thin-layer chromatography for their removal. We have, therefore, turned our efforts to a new chromatographic approach for the isolation of pure preparations of streptococcal glycolipids on columns of silicic acid using the following eluants: chloroform, chloroform-acetone (1:1), acetone, chloroform-methanol (9:1), chloroform-methanol (1:1), and methanol. A unique feature of this procedure is that all separation, concentration, and purification operations are carried out with the original crude chloroform-methanol (2:1 v/v) extract without involving washing with water of the original material or chromatographing the CM 2:1 extract through Sephadex columns to remove non-lipid contaminants. Application of this procedure results in the isolation and concentration of a highly purified glycolipid component in the acetone eluant. The separation of the glycolipid from minor impurities was best achieved by two-dimensional chromatography on borate impregnated silica gel plates. The glycolipid, which revealed a homogenous spot when sprayed with periodate-Schiff reagent, was scraped off plates that were not sprayed and extracted with CM 2:1 (v/v). The $R_f$ value on silica gel TLC of the streptococcal glycolipid was 0.38. This homogeneous material was used for our analytical studies on fatty acids, glycerol, and carbohydrate composition.

A sample of the purified streptococcal glycolipid was subjected to mild alkaline deacylation. Fatty acid and a water-soluble glycoside were recovered as previously described.

Immunogenicity in rabbits. Suspensions of lipid-depleted A. laidalwii membranes, "hybrid" reaggregates, and streptococcus Group D F24-L cells were prepared in phosphate buffered saline (PBS). Suspensions of purified streptococcal glycolipid (isolated from F24-L organisms) were prepared in PBS containing 2 mg lecithin per ml. Rabbits were injected intradermally at several sites with a mixture of 2 ml antigen (protein, 0.75 mg/ml glycolipid 0.5 mg/ml) and 2 ml of complete Freund's adjuvant. Three weeks later, the rabbits were similarly immunized except incomplete Freund's adjuvant was used. After an additional three weeks, 2 ml of antigen was injected intraperitoneally. The rabbits were bled three, six, and seven weeks after the first injection.

Major Findings:

All the streptococcal glycolipids prepared by the new procedure showed high titers in the complement fixation test with antibodies raised in rabbits against whole homologous streptococci, and also cross-reacted in the complement-fixation test with heterologous streptococcal hyperimmune sera. The glycolipid isolated from Staphylococcus aureus cross-reacts with streptococcal rabbit hyperimmune sera to Groups A and D, and the glycolipid from Streptococcus pyogenes Group A 64x392 type I-L form possesses cross-reactive antigenic determinants that react with antiserum to Mycoplasma pneumoniae. However,

the glycolipids isolated from Lactobacillus pifidus and Micrococcus lysodeikticus showed no cross-reactivity in the complement-fixation test with antisera to streptococci. Hydrolysis of the latter two glycolipids revealed that the immunodeterminant groups in the molecule are mannosyl units, while the immunodominant groups of the streptococcal glycolipid are glucosyl units.

The sera of the immunized rabbits were inactivated at 56 C for 30 minutes and tested for the presence of complement-fixing antibodies with the different antigens used in the immunization schedule. The preimmunization sera were either negative or showed very low titers. The reaggregate of streptococcal glycolipid and A. laidlawii membrane protein proved to be effective in eliciting the production in rabbits of antibodies reacting with the glycolipid hapten (first bleeding). The glycolipid itself did not produce any CF antibodies which reacted with any significant titer with the glycolipid. The reaggregate, glycolipid and whole cell vaccine gave high titers in the CF test with the antisera to the whole-cell vaccine.

Analytical chemical characterization of the streptococcal glycolipid.

Oxidation of the glycoside (obtained by mild alkaline deacylation) with 20 nM sodium periodate for 20 hours revealed a 1 → 3 linkage between the glucose residues. Acid or enzymatic hydrolysates of purified glycolipid showed only glucose present in the molecule. Further analytical data, which shows the serologically active glycolipid to be a diglucosyl diglyceride is presented in the following table:

Analytical Data for the Serologically Active Streptococcal Glycolipid

| Constituent | Found | Theory |
|---|---|---|
| Carbon % | 65.10 | 64.7 |
| Hydrogen % | 9.83 | 10.2 |
| Glucose % | 34.7 | 38.0 |
| Glycerol % | 9.3 | 9.7 |
| Fatty acid methyl esters % | 63.7 | 63.0 |
| Molar ratio: glucose-glycerol-ester | 2.00:1.05:2.10 | 2:1:2 |

The molar ratios were calculated for a diglucosyl distearic molecule, $C_{57}H_{96}O_{15}$ with a molecular weight of 948.6.

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Reaggregation. It has previously been shown that purified glycolipids by themselves are poor immunogens. In order to render them more immunogenic, they were mixed with lipid-depleted Acholeplasma laidlawii membrane protein to produce a "hybrid" membrane reaggregate (see Project 64C, 1971-72).

In earlier experiments, such attempts failed. Since then, we have provided the proper reaggregating conditions (30 nM $Mg^{++}$ instead of 20 nM $Mg^{++}$) and produced a hybrid reaggregate of pure streptococcal glycolipid (isolated from F24-L organisms) with A. laidlawii membrane protein, as shown by electron microscopy. The reaggregate, which formed during dialysis, was collected by centrifugation at 36,000 x g for 90 minutes, taken up in deionized water, and protein content determined by the method of Lowry.

New procedure. Because of the large number of paper chromatograms developed in our laboratory for quantitative carbohydrate analysis, and the objectionable odor of the volatile toxic N-butanol used as the solvent for the analine hydrogen phthalate in the color development of the carbohydrate spots, we developed a new sensitive and specific reagent, aniline malonate, for detection of the carbohydrates on the paper chromatograms. The reagent was found to be soluble in an innocuous solvent like ethanol and produced more compact color spots with very little background color.

Significance to Bio-Medical Research and the Program of the Institute:

No evidence is yet available concerning the function of the glycolipids. The compounds may be involved in cell wall synthesis, polysaccharide synthesis or concerned in the substrate transport through membrane of glucosyl groups between the absorbed lipids and the pool of intermediates in carbohydrate synthesis in streptococci.

The widespread occurrence of these glycolipid antigens in the streptococci makes it probable that the compounds fulfill a structural role and are also of some immunological significance. Specific antisera to streptococcal glycolipids may serve as a valuable tool in membrane biochemistry studies of these organisms. Further use can be made of the antiglycolipid antisera in search of serologically related glycolipids in human brain and lung tissue, red blood cells and other microorganisms.

Proposed Course of Project:

Extraction of glycolipids from additional streptococcal groups and other gram-positive and gram-negative bacteria will be continued. Studies on the comparison of the fundamental structural similarities of the various glycolipids and their antigenic determinants, which may be responsible for the cross-reactions encountered in serological tests, will be carried out. Continuation of our studies with "hybrid" reaggregates is in progress.

Honors and Awards:

Invited to participate at the 8th Annual Meeting of the Canadian Society of Chemotherapy, Quebec City, Canada, July 6-7, 1972. Presented a paper, "Potential Antimalarial Agents IV Derivatives of 2-Chloro-4-(p-nitrophenyl-sulfonyl) acetanilide."

Invited and participated at the Schistosome Antigen Workshop held March 7, 1973 at the Naval Medical Research Institute, Bethesda, Md.

Continuing membership: Toxicology Information Program (TIP) at the National Library of Medicine. On roster of Authorities in Specialized Subtopics in Toxicology.

Publications:

King, J. R., Prescott, B., and Caldes, G.: A comparison of the carbohydrates of Streptococcus faecium F24 and its stable L phase variant. J. Bacteriol.(in press)

Caldes, G., and Prescott, B.: A comparative study of certain aniline salts used in the determination of carbohydrates by spot elution chromatography. J. Chromatography (in press)

Serial No. NIAID 65
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Electron microscopy of bacteria in relating structure and function

Previous Serial Number: Same

Principal Investigator: Dr. Roger M. Cole

Other Investigators: Mr. Terry J. Popkin
Dr. Armen Kurkdjian (Guest Worker, Institute Pasteur, February - June, 1973)

Cooperating Units: University of Arizona, College of Liberal Arts, Dept. of Microbiology and Medical Technology, Tucson (Dr. Neil H. Mendelson, Dr. John N. Reeve)

University of Rochester, School of Medicine and Dentistry, Dept. of Microbiology (Dr. Anadi S. Chatterjee, Dr. Frank E. Young)

University of Massachusetts Medical School, Dept. of Microbiology, Worcester (Dr. Donald J. Tipper, Dr. Carl M. Good)

I. N. R. A., Centre de Recherches de Bordeaux, France (Dr. Jean-Marie Bové)

Veterans Administration Hospital, Baltimore (Dr. Willy N. Pachas)

Central Public Health Laboratory, Colindale, London (Dr. W. R. Maxted)

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 21/12 | - | 4/12 | 25/12 |
| Professional | 18/12 | - | 4/12 | 22/12 |
| Other | 3/12 | - | - | 3/12 |

Project Description

Objectives:

To examine the ultrastructure of microorganisms, in self-generated and collaborative studies, with particular emphasis on bacterial cell walls and membranes, their related antigens, and aspects of their participation in growth, division, genetic interchange, bacteriophage absorption, and the like.

Methods Employed:

Standard or newly-developed methods of bacteriology, serology, phage virology, microbial genetics, biochemistry, and electron microscopy. When appropriate, special methods of antibody labelling, cytochemistry, or other techniques of marking for electron microscopy.

Major Findings:

(A) Bacillus subtilis mutants

1. A new, non-temperature-sensitive mutation causing a Class IV division defect (alteration of the site of cross-wall formation) was transferred to an isogenic strain, alone or with the tag-1 temperature-sensitive locus previously described. The new locus (Div. IV) causes "mini-cell" formation at poles of long cells, singly or in successive or multiple division. "Mini-cells" so produced have been purified and concentrated. They are motile and respire, but fail to grow. They do not autolyze, as do parent cells, at 37 C, but are lysozyme-sensitive. Radiolabelling methods and electron microscopy (EM) show them to be devoid of DNA (nucleoids). EM demonstrates normal wall and other ultrastructure, even at 45 C, of mini-cells from parents with or without the tag-1 locus--although at this elevated temperature, both autolyze. The failure to find ultrastructural evidence of cell wall or other changes at 45 C shows that the phenotype cannot be expressed in mini-cells from tag-1-containing parents, and indicates the lack of genetic information and resultant corrected synthetic processes. Mini-cells provide excellent material for similar separation, and metabolic and ultrastructural examination, of various biosynthetic processes. (Collaborators Mendelson and Reeve)

2. A mutant (RUB 1402) deficient in D-alanine-L-alanine racemase, was examined at intervals of incubation. The cells lack the dense periplasm seen in most bacilli, and the outstanding changes seen are: (a) accumulation of a homogeneous material in nucleoids, beginning at 20 minutes; (b) expansion of periplasm containing homogenous material, seen only at incipient, initiated, or completed cross-wall sites in survivors at 70 minutes; (c) conversion of vesicular mesosomes to lamellate, expulsion of these accompanied by peripheralization of nucleoids, and progressive lysis of many cells. Considerable work remains to be done, but the failure of wall-incorporated D-alanine to "turn over" in the absence of synthesis (as shown by our collaborators) suggests a relation of D-alanine-containing precursors to the periplasmic and intranucleoid changes seen. (Collaborator - Young)

(B) Staphylococcus aureus mutants

We previously examined a mutant (tar-1) which is deficient in cell wall teichoic acid and active autolysin. The presence of 1 M NaCl in growth media at 37 C resulted in a marked loosening and fraying of wall, as seen by electron microscopy (EM): in buffer, complete loss of wall was seen concomitant with gross lysis, as measured turbidimetrically. These results are interpreted as stimulation of autolysis by NaCl, which is particularly enhanced in buffer where autolytic activity is not balanced by synthesis. In contrast, TOF (for temperature-sensitive osmotically-fragile) mutants examined this year grow normally at 30 C, but fail to grow at 43 C and lyse unless 1 M NaCl is present. EM showed, in the absence of NaCl, a widening of the cell wall profile which became progressively looser, frayed, and broken--leading to lysis. When NaCl was present, the wall was thin and dense, and all cells remained intact. These results suggest an unbalanced situation in which a defect in wall synthesis is conditionally expressed at the restrictive temperature and is corrected by the presence of NaCl. Our collaborators have now shown that the biochemical defects in these mutants fall into 4 classes, which can lead to defects in synthesis of the peptides or peptide cross-bridges that comprise part of the rigid cell wall peptidoglycan. The defect can be in one of three ligases which bind amino acids (L-alanine, D-glutamine, or L-lysine) into the peptide side chains; or in synthesis of the D-alanine-D-alanine which comprises part of the interpeptide bridges.

At 30 C, TOF mutants also grow in the presence of NaCl, but EM shows considerable widening and loosening of wall. The same is seen in the parent strain, and to a lesser extent at 43 C; similar changes in presence of NaCl occur in the parent of tar-1 and some other "wild-type" strains at normal incubation temperatures. These latter also show walls of increased thickness and density if grown overnight or at elevated temperature, and these changes are reversed or prevented by 1 M NaCl. The results to date suggest that autolysis normally plays a role in regulation of cell wall architecture and that it can be stimulated by 1 M NaCl. When, as in tar-1, wall is deficient in teichoic acid, which has a function regulatory to autolysis, the NaCl-stimulated autolysis becomes predominant and, in the absence of synthesis can lead to complete wall degradation and lysis. When, as in TOF, the mutant defect is in assembly of the peptidoglycan and is conditionally expressed, incubation in the restrictive condition leads to lysis because normal, non-stimulated, autolytic activity overbalances the defective synthesis. The role of NaCl in preventing this lysis is unknown, but the maintenance of normal wall architecture in its presence suggests that processes other than simple osmotic support are involved. (Collaborators - Chatterjee; Good and Tipper)

(C) Ultrastructure of first characterized mycoplasma of plant origin

Considerable effort was devoted to examining the morphology of Spiroplasma citri, the first mycoplasma from plant (diseased orange trees) to be cultured and characterized as a new genus and species (See also Project NIAID 207). By dark-field and phase microscopy from liquid (but not from agar) cultures, the organism is a motile, helical filament with associated blebs; old cultures

lose both motility and helical shape. Electron microscopy (EM) revealed the absence of cell wall and of organelles that might account for shape and motility; and also showed consistent, but probably defective, infection by a tailed bacteriophage in both isolates examined. We found that helical shape was lost by many treatments; its demonstration required ammonium molybdate instead of sodium phosphotungstate for negative-staining EM; and primary fixation by glutaraldehyde without the usual buffer for sections. These successful treatments seem associated with maintenance of the high osmolality of the culture medium.

Spiroplasma also possesses a nap-like or fuzzy outer layer (not a cell wall) to which the phage appears to attach. Frequently, but not invariably, another interrupted layer is seen abutting the inner side of the single membrane that bounds the organism; this may represent phage-associated structures. On or in swollen or round bodies that appear to represent aging organisms, there are striated bodies composed of stacks of doughnut-like stellate units, which can be disaggregated to some extent by treatment with water and/or pronase. The nature of these and of outer layer is being explored. Preliminary examination by gas-liquid chromatography showed the absence of hexosamines or other known cell wall constituents. Two additional strains, isolated by other workers in locations other than those of the original isolate, have been examined and are structurally and culturally identical and both phage-infected. Also, we have examined what appears to be a closely similar organism, formerly mistakenly called a spirochaete, which occurs in and and affects the sex ratio of progeny of certain species of Drosophila. These organisms offer unique opportunities for studies of membranes and phage-host interactions in an important and apparently widespread new group of microorganisms, and future efforts will be in these directions. (Collaborator - Bové; Tully)

(D) Electron microscopy in support of other projects, or in preliminary stages

1. In supplementation of basic studies of phage-host interaction in Group H streptococci, the binding of phage 227 to purified cell walls and stages of its vegetative development in a sensitive strain (WE4), are being examined. (Project No. NIAID 65A-2; Nugent)

2. A fimbriate surface layer was observed, by negative staining, on 10 of 23 "Group H" strains. The 3 most transformable strains, lack this layer and are not lysogenized; but its presence in the others does not correlate with attributes such as lysogeny, serological cross-reactions, nor species as determined by cultural and biochemical tests.

3. A preliminary study was made of effects of growth-inhibiting concentration of several antibiotics on ultrastructure of Staph. aureus 6538P. All (mitomycin C, novobiocin, rifampin, erythromycin, fusidin, and penicillin G) caused thickening of cell wall and some distortion of division processes. Mitomycin and rifampin induced visible bacteriophage, and these as well as novobiocin caused the appearance of undefined, radially-arranged structures apparently emanating from membrane. Excessive accumulations of extracellular excess wall material appeared in the presence of novobiocin and rifampin. Anticipated alterations in frequency or structure or mesosomes were not seen. (Popkin)

4. Examinations of the ultrastructure of Erysipelothrix rhusiopathiae, its L form, and revertants, were made. The parent and revertant forms of this pathogen are typical Gram-positive bacteria with large mesosomes and the L forms show no features distinguishing them from others. (Collaborator - Dr. Pachas)

5. Studies were begun on the ultrastructure of strains of Group A streptococci characterized as to the presence or absence of M protein, serum-opacity factor (SOF), or combinations thereof. The relation of M and SOF in difficultly typable and poorly antigenic strains is not understood, and it is possible that ultrastructural studies may localize the presumed surface distribution, and determine the nature of SOF. (Collaborator - Dr. W. R. Maxted)

6. The effects of citrate on the ultrastructure of streptococcus Challis (Group H) grown in optimal $CO_2$ concentrations, are under study. Early results show enlargement and bizarre cellular morphology when citrate is present. (Project No. NIAID 61; Repaske)

7. Examination of plant tumors (crown-gall) initially induced by Agrobacterium tumefaciens was initiated. The effort is to obtain ultrastructural evidence for the presence and localization of the Agrobacterium phage, or its DNA, within tumor cells--to confirm sporadic reports of phage isolation by bacteriologic methods, in the absence of bacterial isolations. Suspicious but non-definitive bodies were seen in cell mitochondria, and present efforts are directed to examination of isolated mitochondria and development of methods for labelling phage, phage DNA, or products coded by the DNA. (Guest worker Dr. Kurkdjian and Dr. Parsons)

8. Ultrastructural studies were made to compare mating strains of Emmonsiella capsulata and Ajellomyces dermatitidis; to characterize the asexual spores of E. capsulata and conidia from human and canine isolates; and to compare normal and amphotericin B-resistant Aspergillus. (Project No. NIAID 77-1-F; 77-1-G; Drs. Kwon-Chung and Kim; Dr. Kurkdjian)

9. Continuous EM monitoring is carried out on membrane and mesosome preparations (Project No. NIAID 63B, Theodore), reaggregate hybrid membranes (Project No. NIAID 64D, Prescott), and new strains of Spiroplasma and other mycoplasmas (Project No. NIAID 207, Tully).

Significance to Bio-Medical Research and the Program of the Institute:

The purpose of our major studies at present is to clarify the relationships among bacterial growth and division, presence or absence of cell wall constituents, and cell wall autolytic enzymes. To this end, we are comparing ultrastructurally, various mutants of Gram-positive bacteria to one another and to normal or "wild-type" organisms--when grown under different conditions. The results show that mutations, which can be specifically localized and transferred to a variety of genetic backgrounds in other strains, can effect production of specific cell wall components (for example, teichoic acid). Its lack, in turn, is associated with defects in autolytic activity and with concomitant ultrastructural changes in cell size, shape, division, and nature of the cell wall--in both Bacillus and Staphylococcus. Conditions which

stimulate or inhibit autolytic activity have been shown to produce characteristic cell wall, other morphologic, and growth, changes--even in wild-type bacteria, although to a lesser extent than in mutants. We are, therefore, approaching definitive answers to the genetic basis for, and intermediary regulatory factors in, the control of growth and division among walled microorganisms which normally maintain a characteristic size and shape.

Proposed Course of Project:

We expect to continue study of ultrastructure of normal and mutant Gram-positive bacteria and of isolated bacterial components, and to emphasize more the structural-functional relationships in streptococci.

Honors and Awards:

Invited Seminars and Lectures

"Ultrastructural and other characterization of a new, helical, motile, bacteriophage-infected mycoplasma cultured from a disease of orange trees." Department of Microbiology, University of Massachusetts, Amherst, Nov. 16, 1972.

"Bacterial cell wall mutants: Ultrastructural and other changes. (a) Bacilli; (b) Staphylococci." 1972-73 Lecture Series on Biological Regulation, Thomas Jefferson University, College of Graduate Studies, Philadelphia, Jan. 8-9, 1973.

"Ultrastructure of the agent of 'stubborn' disease," Conference on Mycoplasma and Mycoplasma-like Agents of Human, Animal and Plant Diseases, N. Y. Academy of Sciences, Jan. 11, 1973.

"Bacterial Ultrastructure," Advanced Topics in Microbiology, FAES Graduate School, NIH, February 7, 1973.

"The problem of bacterial shape and partition: an examination of ultrastructural and associated changes in cell wall and division mutants," Dept. of Microbiology, Medical College of Wisconsin, Milwaukee, Feb. 27, 1973.

"Ultrastructural and other features of a new, helical, mycoplasma-like organism from diseased orange trees," Dept. of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, March 5, 1973.

"Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma-like organism cultured from a disease of orange trees," Division of Biological Sciences, University of Florida, Gainesville, March 20, 1973.

"Ultrastructure of teichoic acid-deficient and other mutants of staphylococci," Internat. Conference on Recent Advances in Staphylococcal Research, N. Y. Academy of Sciences, June 11-13, 1973.

Participant, First International Meeting on Bacterial Transformation, Oeiras, Portugal, Aug. 31 - Sept. 2, 1972.

Leader: NIAID Group to visit biomedical institutions in Poland, Sept. 3-17, 1972.

Project Officer, NIAID Research Contract PH 43-68-83, University of Chicago.

Member, Site Visit Team to Department of Microbiology , University of Minnesota Medical School, Extramural Programs, NIAID, April 2, 1973.

Member Ad Hoc Review Committee for Special Emphasis Program on Streptococci, Extramural Programs, NIAID, April 30, 1973.

Appointed to Editoral Board, Infection and Immunity, November 1972.
Elected to membership, Infectious Disease Society of America, Sept. 1972.

Publications:

Glick, A. D., Ranhand, J. M., and Cole, R. M.: Degradation of Group A streptococcal cell walls by egg-white lysozyme and human lysosomal enzymes. Infect. Immun. 6: 403-413, 1972.

Parsons, C. L., Colon, A. E., Leonard, C. G., and Cole, R. M.: Isolation of bacteriophages from "Group H" streptococci. J. Virol. 9: 876-878, 1972.

Ranhand, J. M., and Cole, R. M.: Lysis of streptococci by an extracellular lysin produced by competent Group H streptococci strain Challis. J. Gen. Microbiol. 71: 199-202, 1972.

Mendelson, N. H., and Cole, R. M.: Genetic regulation of cell division initiation in Bacillus subtilis. J. Bacteriol. 112: 994-1003, 1972.

Reeve, J. N., Cole, R. M., and Mendelson, N. H.: Cell morphology of Bacillus subtilis: The effect of genetic background on the expression of a rod⁻ gene. Molecular and General Genetics 119: 11-26, 1972.

Parsons, C. L., Ranhand, J. M., Leonard, C. G., Colon, A. E., and Cole, R. M.: Inhibition of transformation in Group H streptococci by lysogeny. J. Bacteriol. 113: 1217-1222, 1973.

Saglio, P., Lhospital, M., Lafleche, D. Dupont, G., Bove, J. M., Tully, J. G., and Freundt, E. A.: Characterization of Spiroplasma citri, gen. nov, sp., nov.: A new mycoplasma associated with "Stubborn" disease of citrus. Internat. J. Systematic Bacteriol. (In press)

Cole, R. M., Tully, J. G., Popkin, T. J., and Bove, J. M.: Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma (Spiroplasma citri gen. nov., sp. nov.) cultured form "Stubborn" disease of citrus. J. Bacteriol. (In press)

Reeve, J. N., Mendelson, N. H., Coyne, S. I., Halloch, L. L., and Cole, R. M.: Mini-cells of Bacillus subtilis. J. Bacteriol. (In press)

Cole, R. M., Tully, J. G., Popkin, T. J., and Bove, J. M.: Ultrastructure of the agent of "Stubborn" disease. Ann. N. Y. Acad. Sci., N. Y. (In press)

Serial No. NIAID 65A
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the genetics of streptococci

Previous Serial Number: Same

Principal Investigator: Alba E. Colón

Other Investigators: Carmen G. Leonard
C. Lowell Parsons
Roger M. Cole

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 21/12 | - | - | 21/12 |
| Professional | 12/12 | - | - | 12/12 |
| Other | 9/12 | - | - | 9/12 |

Project Description:

Objectives:

To develop and apply systems of genetic exchange, namely transformation, transduction and transfection, in streptococci. The work also includes search for methods to detect or select transductants to various bacterial antigens such as M protein, C-carbohydrate, hemolysins, etc. Also, to continue studies on other antibiotic markers, as well as lysogenic conversion.

Methods Employed:

The usual methods in microbial genetics, bacteriophage isolation and identification were employed.

Major Findings:

Last year we reported isolation of bacteriophages from Group H streptococci (Project No. 65A-1, Parsons). Using these phages and a Group A transducing

phage (FW), we have tried to transduce antibiotic markers within Group H streptococci. To obtain transducing phages as well as recipient strains, we used both the plate and tube transducing methods. The following conditions were tested for obtaining possible transducing phages: (1) induction with mitomycin-C of streptomycin resistant lysogenic strains, and (2) propagation of Channon and FW227 phages at different temperatures in strain Wicky resistant to one or several antibiotics (namely, rifampin, erythromycin, and streptomycin). The resulting phages were tested for capability to transduce these antibiotic markers to phage-sensitive strains of Group H. To obtain transducible (recipient) strains, we tested strains SBE, Wicky, and Challis before and after treatment with nitrosoguanidine. After extensive screening, transduction to the streptomycin marker was finally obtained in four isolates obtained from strain SBE after treatment with nitrosoguanidine. The transducing phage used was FW227 propagated at 37 C in strain Wicky resistant to streptomycin. The transducing efficiency obtained in SBE-2 is $5 \times 10^{-6}$. At present, we are trying to obtain transduction in other Group H strains, such as Wicky and Challis.

Significance to Bio-Medical Research and the Program of the Institute:

With the present finding of transduction in Group H streptococcus, we have now obtained for the first time, in one group of streptococci the three main systems of genetic exchange; namely, transformation, transfection, and transduction. This result now enables a multi-pronged attack on mechanisms of genetic variation in streptococci, and on genetic control of streptococcal structure, chemical composition, virulence, pathogenicity, and taxonomic position.

Proposed Course of Project:

To continued investigations on the genetics of streptococci. The work will include: (1) studies on transduction of different markers, (2) search for methods for the selection of transductants to antigenic markers, and (3) inter and intra-group transduction of antigenic markers.

Honors and Awards:

None

Publications:

Colon, A. E., Cole, R. M., and Leonard, C. G.: Intergroup lysis and transduction by streptococcal bacteriophages. J. Virol. 9: 551-553, 1972.

Parsons, C. L., Colon, A. E., Leonard, C. G., and Cole, R. M.: Isolation of bacteriophages from Group H streptococci. J. Virol. 9: 876-878, 1972.

Parsons, C. L., Ranhand, J. M., Leonard, C. G., Colon, A. E., and Cole, R. M.: Inhibition of transformation in Group H streptococci by lysogeny. J. Bacteriol. 113: 1217-1222, 1973.

Serial No. NIAID 65A-1
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Group H streptococcal bacteriophages: effects on genetic systems of transformable streptococci.

Previous Serial Number: Same

Principal Investigator: Dr. C. Lowell Parsons

Other Investigators: Dr. Roger M. Cole
Miss Alba E. Colon
Mrs. Carmen G. Leonard
Dr. Jon M. Ranhand

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 26/12 | - | - | 26/12 |
| Professional | 17/12 | - | - | 17/12 |
| Other | 9/12 | - | - | 9/12 |

Project Description:

Objectives:

To examine the reasons lysogenic strains of Group H streptococci, previously highly competent for DNA mediated transformation, no longer transform in the lysogenic state. To also attempt recovery of virulent phages which will infect these lysogenic Group H strains. To develop a system of transduction in these strains in order to explore the relationships of phage-mediated transduction, phage DNA-mediated transfection, and lysogeny to genetic competence.

Methods Employed:

Standard methods of bacteriological culture and bacteriophage assay. Nitrosoguanidine, nitrous acid and ultraviolet light were empolyed as mutagenic agents and mitomycin C was used to induce the vegatative phage propagation in bacterial strains.

Major Findings:

The isolation of several bacteriophages infecting the Group H streptococci was reported last year. We have now been able to successfully concentrate the phages and purify their DNA. This DNA transfects strains Challis and Wicky with 1.0 ugm yielding about 2 x $10^4$ PFU's.

We have shown that two Group H streptococcal strains highly competent for DNA mediated transformation, transform three orders of magnitude less when they are lysogenized. This phenomenon has been reported in B. subtilis, but unlike that system, we have found that (1) the small percentage of cells that do transform are cured of the phage suggesting the phenomenon is reversible, and (2) that lysogenic cells do not bind DNA reversibly or irreversibly (DNAase resistant form).

These lysogenic cultures were examined to determine why they no longer transform; no DNAases are produced which will inactivate transforming DNA and as mentioned above, they make no sites which will bind DNA either reversibly or irreversibly (DNAase resistant binding). A mutant lysogenic bacterial strain has been recovered which is able to transform and when its phage is used to lysogenize competent cultures, they no longer transform. Further, when the DNA of this mutant is used to transform competent strains of Wicky or Challis, there are no cells which recombine the prophage locus and become lysogenic but transfection does occur. This suggests that the DNA of the phage is not integrated with the bacterial chromosome and may exist as a plasmid.

Bacteriophages grown in strain Wicky have been found to transduce strain SBE-2, another Group H bacterium which is highly competent for DNA mediated transformation. (See Project NIAID 65A)

Using a variety of mutagens, we were unable to obtain any clear plaque mutants of the Group H bacteriophages which we isolated and reported last year. We also obtained 13 additional strains of Group H streptococci but found no virulent phages among them.

Significance to Bio-Medical Research and the Program of the Institute:

Until we recovered bacteriophage infecting Group H streptococci, there was no one system in streptococci in which transformation, transfection, and transduction could be performed. We now can use these methods of recombination to study the relationship that these genetic exchange mechanisms have to each other and have a greater variety of approaches to studying the nature of competence and the problems of genetic exchange. As regards to the inhibitory effect of lysogeny of transformation, we find it is secondary to a loss of, or an alteration of, DNA binding sites; this lysogenic inhibition of transformation is seen in B. subtilis, but unlike streptococci, there is no loss of the ability to bind DNA. We hope to be able to look at this unique phenomenon at a molecular level.

Proposed Course of Project:

We plan to use the three genetic tools now available to clarify genetic mechanisms of competence in general, and streptococci in particular. We want to further study the mechanisms of why lysogenic cells don't make intact DNA binding sites and to study recombination in lysogenized cells via transduction. We will look at the fate of phage DNA during transfection and also try to obtain virulent phage mutants to aid these studies.

Honors and Awards:

None

Publications:

Parsons, C. L., Ranhand, J. M., Colon, A. E., Leonard, C. G., and Cole, R. M.: Inhibition of transformation in Group H streptococci by lysogeny. J. Bacteriol. 113: 1217-1222, 1973.

Parsons, C. L., and Cole, R. M.: Transfection of Group H streptococci. J. Bacteriol. 113: 1505-1506, 1973.

Serial No. NIAID 65A-2
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Investigation of Group H streptococcal temperate bacteriophages: Fundamental characteristics of phage-host interaction; Fate of cellular DNA during vegetative infections

Previous Serial Number: None

Principal Investigator: Dr. Kenneth Nugent

Other Investigators: Dr. Roger M. Cole

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 12/12 | - | - | 12/12 |
| Professional | 12/12 | - | - | 12/12 |
| Other | - | - | - | - |

Project Description:

Objectives:

This project is a long-term investigation of Group H streptococcal temperate bacteriophages. The initial goal is to study the fundamental characteristics of phage-host interaction and the fate of cellular DNA during vegetative infection.

Methods Employed:

Routine bacteriological and bacteriophage techniques. Routine procedures for extraction and analysis of DNA by transformation and sucrose gradients. All work was done with phage $\phi$227 and indicator strain WE 4 (a derivative of strain Wicky).

Major Findings:

A substantial portion of the early work has been preliminary in nature and devoted to adapting routine techniques to Group H Streptococci and their bacteriophage.

These phage adsorb irreversibly to cellular debris and this prevents preparation of high titer stable phage stocks. The following methods of purification were tried and discarded:

1. high-speed centrifugation
2. differential centrifugation
3. antisera to cell wall to block adsorption
4. ammonium sulfate precipitation
5. methanol precipitation
6. polyethylene glycol-sodium chloride precipitation

The most efficient procedure was mixing phage lysates with diatomaceous earth, followed by filtration and phage collection with high-speed centrifugation. This method results in stable phage stocks with titers of 1-4 x $10^{11}$ ml.

The routine media for Streptococci are enriched and undefined. This makes radioactive precursor incorporation studies and media composition studies impossible. A reasonably flexible chemically defined synthetic media with AOAC synthetic Broth for the base was developed. This media supports excellent growth of Group H Streptococci (mass doubling time 47 - 50 minutes), transformation, and phage growth. In addition, radioactive thymidine is incorporated into cellular DNA.

Fundamental characteristics of phage-host interaction: Adsorption is rapid ($K = 0.346 - 0.692\ B^{-1}$ min. $^{-1}$ at MOI <0.1) and is 95% to 99% complete by seven to eight minutes. Adsorption occurs in the absence of calcium and in the presence of 0.01 M citrate. The following cation concentrations did not significantly affect the rate of adsorption: 1 to 500 mM Mg++, 1 mM Zn++, 1 M K+ and 1 M Na+.

| | Latent Period | Rise Period | Burst/Infective Center |
|---|---|---|---|
| P Broth | 42-45 | 45-50 | 25-125 |
| AOAC Media | 50-55 | 55-65 | 10-27 |

The burst size in enriched media is reduced by inactivation of an unknown heat labile compound. Citrate inhibits productive infections; this effect is due to the inhibition of cell lysis. Cell killing following phage infection is greater in rich media, as expected, but also is increased by increasing multiplicities.

The following information suggests that phage "take over" during vegetative infection does not involve significant alteration of cellular DNA. Hydroxyurea at 0.01 M concentration completely inhibits phage replication. Tritium labeled cellular DNA does not become acid soluble during the course of phage infection. When host DNA is extracted at intervals during phage infection, the frequency of cotransformation of two linked markers is constant.

Significance to Bio-Medical Research and the Program of the Institute:

The work to date is preliminary in nature and represents an initial attempt to investigate a new temperate phage system. Some of the problems associated with these systems serve as models for understanding multiple pathway responses to challenge from biological agents. These problems include the sequence of events by which the phage takes over host cell's metabolism, the factors which influence the lysogeny vs. replication decision, and the mechanism by which the lysogenic state alters the cell's metabolism (e.g., inhibition of transformation). One approach to these problems is the utilization of mutants. The outlook for such studies in Streptococci is now less pessimistic because of recent advances in developing systems of genetic exchange.

Proposed Course of Project:

1. The work concerning the fate of cellular DNA during phage infection will be tentatively concluded after the following studies:

   a. alkaline sucrose gradient analysis of DNA size distribution during the course of infection

   b. electron microscopy to follow the integrity of the nuclear region

   c. the effect of metabolic inhibitors on phage replication

2. Analytical studies of bacteriophage, including electron microscopic examination of the attachment site of phage to purified cell wall, polyacrylamide gel analysis of the number and size of head and tail proteins, and molecular weight and base content determination of phage DNA.

3. A minor project will be a survey of methods for increasing the utilization of thymidine by Streptococci. Preliminary results indicate that deoxyadenosine and uridine inhibit thymidine incorporation by >95%.

Honors and Awards:

None

Publications:

None

Serial No. NIAID 65C
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Competence development and genetic transformation in Group H (and Group A) streptococci: early events

Previous Serial Number: Same

Principal Investigator: Mrs. Carmen G. Leonard

Other Investigators: Miss Alba E. Colon
Dr. Roger M. Cole

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Staff | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 35/12 | - | - | 35/12 |
| Professional | 12/12 | - | - | 12/12 |
| Other | 23/12 | - | - | 23/12 |

Project Description:

Objectives:

To continue to define and understand the events induced by added competence factor(s) which lead to the competent state in Group H streptococci. Also, we have recently initiated efforts to develop a transformation system in Group A streptococci where transformation has never been reported. The availability of this new transformation system would greatly enhance our studies on the mechanism of competence induction.

Methods Employed:

The usual methods in microbial genetics was employed (see previous reports). New techniques were developed for screening of possible transformable mutants among Group A cultures.

Major Findings:

A. Studies in competence development of Group H streptococci.

Previously we described three distinct and separate events involved in competence development. These events are (1) production and release of competence factor (CF); (2) CF binding by non-competent cells; and (3) subsequent induction of competence by the newly cell-bound CF. We have now shown, by the use of inhibitors of protein synthesis, that each of these events requires separate periods of protein synthesis--each of varying length. It was found that, (1) CF production requires less than 30 minutes of uninterrupted protein synthesis; (2) CF binding requires 5 minutes; and (3) competence induction by the cell-bound CF requires about 30 minutes. Therefore, in all, about 60 minutes of protein synthesis is required by CF producing cells (Challis) and about 35 minutes by CF requiring cells (Wicky) for cells to become competent as measured by transformation.

The processes induced by cell-bound CF are not fully understood, but certain new properties, previously described by others as indicating competence, were measured during the above mentioned steps of competence development. Previously, it was reported by others that only competent cells aggregate at pH 2.0 but our results show that this property is related to cell-bound CF and appears prior to competence development. It has also been reported that significant stimulated autolysis appears as competence develops. Although autolysins may be required for competence, our results show that the ability of cultures to autolyze maximally can be diminished by appropriately-timed treatment of pre-competent cells with trypsin without affecting subsequent transformability.

Another property previously described as a measure of competence is the appearance of a new protein band in phenol extracts of competent cells as measured by acrylamide gel electrophoresis. We found that treatment of pre-competent cells with trypsin also prevents the appearance of this new protein band without affecting transformability. Therefore these three properties, although as a rule normally exhibited by competent cells, are not--in their fullest manifestations--essential indicators of competence as measured by transformability. Their roles remain to be elucidated and thus continue to be the subject of our studies.

B. Studies on transformation of Group A streptococci.

There is no known transformation system in Group A streptococci, although we discovered a transduction system. The availability of both systems of genetic exchange would greatly help our understanding of the genetics and pathogenicity of this group, and a new transformation system in streptococci would be of great value in determining the mechanism of competence induction.

We have started to screen cultures of Group A streptococci before and after treatment with mutagenic agents for possible mutants. Transformation tests are being conducted under different cultural conditions such as temperature, pH, presence of metallic ions, use of mutants, etc. Efforts to date have not been successful.

Significance to Bio-Medical Research and the Program of the Institute:

We have been able to further separate and characterize early events involved in competence development in streptococci. We have now a better understanding of the possible roles of CF in competence development and are now able to study each event independent of the other. Any new information on how a bacterial cell can take in extracellular deoxyribonucleic acid and thus be transformed has many applications to our understanding of this genetic exchange in bacterial as well as animal cells. Also the availability of transformation, as well as transduction in Group A streptococci would help in further understanding the genetics and pathogenicity of this group.

Proposed Course of Project:

We plan to continue studying the mechanism of competence induction in Group H streptococci. We hope to determine the nature of the initial binding of CF and of the newly induced proteins. We also plan to expand our previous studies on the effects of lysogenization on transformation in Group H, as a possible way of understanding the phenomenon of competence, (See Project No. NIAID 65A-1, Parsons) and possibly in Group A streptococci.

In addition, we will continue to search for transformable strains in Group A streptococci. These studies in conjunction with transduction studies in Group H and A (See Project No. NIAID 65A, Colon) will furnish the tools and basis for significant studies on the genetics of streptococci.

Honors and Awards:

None

Publications:

Leonard, C. G.: Early events in development of streptococcal competence. J. Bacteriol. (in press)

Parsons, C. L., Colon, A. E., Leonard, C. G., and Cole, R. M.: Isolation of bacteriophages from "Group H" streptococci. J. Virology 9: 876-878, 1972.

Parsons, C. L., Ranhand, J. M., Leonard, C. G., Colon, A. E., and Cole, R. M.: Inhibition of transformation in Group H streptococci by lysogeny. J. Bacteriol. 113: 1217-1222, 1973.

Serial No. NIAID 65C-1
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Competence development and genetic transformation in Group H streptococci: nature and role of autolysins

Previous Serial Number: Same

Principal Investigator: Dr. Jon M. Ranhand

Other Investigators: None

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 24/12 | - | - | 24/12 |
| Professional | 12/12 | - | - | 12/12 |
| Other | 12/12 | - | - | 12/12 |

Project Description:

Objectives:

To define the role of autolysins in the development of competence in Group H streptococci.

Methods Employed:

Standard microbiological methods are used for culturing and maintaining cell stocks. Autolysis of competent cultures is measured as a decline in optical density (in a buffer), as defined in last year's report.

Major Findings:

Competence is the ability of bacterial cells to react with exogenously supplied deoxyribonucleic acid (DNA) and thereby become genetically changed or transformed. The nature of competence is not fully understood in streptococci nor in any of the other known transformable bacteria, i. e., pneumococci,

Haemophilus. Evidence presented in an earlier report showed that competence development in Group H streptococci involved the induction of a new protein (detected after polyacrylamide gel electrophoresis of phenol-acetic acid extracts of whole cells) and an autolytic enzyme. The nature of the protein has not yet been determined.

After discovering the unique lytic system in Group H streptococci, I undertook a survey to determine whether the competent cell associated lysin was specific for lysing (i) Group H organisms, (ii) streptococci from other serological groups, or (iii) bacteria from other genera. The lysin, from culture supernatant fluids of competent strain Challis, was shown to be active on some strains of streptococci from a few serological groups (A, E, H, and K, one strain) and inactive on strains from other groups (B, C, D, F, G, K, one strain, L, M, N, P, Q, R, and S) as well as other Gram positive and Gram negative organisms. At least two strains from each of the above serological groups (except one only from Group E) were tested.

This list has now been extended to include 17 strains of Group H streptococci. The susceptibility of these cells to the lysin varied from high to low. The usual transformable strains (Wicky and Challis) are most susceptible. However, this is not related to transformability. The least susceptible and one insusceptible strain are those that appear not to be Group H strains as judged from recent serologic evidence.

These combined observations suggest that some serological groups of streptococci (A, E, H, and K, one strain) share common cell wall constituents with the Group H cells, and not all Group H cells share them equally. These differences that do exist can be resolved only after determination of the nature of the lysin and its substrate.

In other studies that pertain primarily to the Group H strains, it was verified that strains that do transform, either spontaneously or after addition of competence inducing factors, autolyze to a high degree; strains that do not have the potential for developing competence do not. Transformable strains that do not autolyze have not been found.

Other findings germane to the competence picture as it exists in the Group H streptococci are: (1) all competent populations release into their menstruum a lysin that is capable of lysing noncompetent Wicky cells. This lysin may be the same one that causes autolysis of competent cells, and (2) the degree of autolysis and the degree of competence attained in the population are correlated.

Significance to Bio-Medical Research and the Program of the Institute:

The nature of competence is elusive. My present work has aided in defining this remarkable phenomenon. Since the Group H streptococci produce a soluble factor (CF) that induces the competent state in otherwise noncompetent cells, the degree or extent of competence can be controlled, and therefore, the events that lead to its full attainment can be studied.

Proposed Course of Project:

I plan to investigate in detail the nature and location of the newly synthesized competent cell associated protein and to investigate the role played by the autolysin in competence development. In addition, I will attempt to purify the autolysin, which is unique in that it attacks only newly synthesized cross wall.

Honors and Awards:

Lecturer, Foundation for Advanced Education in the Sciences.

Participant at the First International Transformation Meeting in Oeiras, Portugal (1972).

Presented a paper at the 72nd Annual Meeting of the American Society for Microbiology in Philadelphia, Penn. (1972).

Publications:

Ranhand, J. M., and Cole, R. M.: Lysis of streptococci by an extracellular lysin produced by competent Group H streptococcus strain Challis. J. Gen. Microbiol. 71: 199-202, 1972.

Glick, A. D., Ranhand, J. M., and Cole, R. M.: Degradation of Group A streptococcal cell walls by egg white lysozyme and human lysosomal enzymes. Infection and Immunity 6: 403-413, 1972.

Parsons, C.L., Ranhand, J. M., Leonard, C. G., Colon, A. E., and Cole, R.M.: Inhibition of transformation in Group H streptococci by lysogeny. J. Bacteriol. 113: 1217-1222, 1973.

Ranhand, J. M.: Autolytic activity and its association with the development of competence in Group H streptococci. J. Bacteriol. (in press)

Serial No. NIAID 65F
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The cell wall constituents found in a stabilized L form

Previous Serial Number: Same

Principal Investigators: James R. King and Benjamin Prescott

Other Investigators: George Caldes

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | - | - | - | - |
| Professional | - | - | - | - |
| Other | - | - | - | - |

Project Description:

See 1972 Annual Report.

Project has been terminated.

Serial No. NIAID 65G
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Characterization of biophysical and physiological properties of L forms

Previous Serial Number: Same

Principal Investigator: James R. King

Other Investigators: None

Cooperating Units:
1. Dr. Zell A. McGee
Associate Professor of Medicine
George Hunter Laboratories
Vanderbilt Medical School
Nashville, Tennessee

2. Gunter F. Thomas
Laboratory of Viral Diseases, NIAID

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 10/12 | - | - | 10/12 |
| Professional | 10/12 | - | - | 10/12 |
| Other | - | - | - | - |

Project Description:

Objectives:

To define the biophysical properties of L forms of bacteria; and to study the physiological response of L forms to *in vivo* environments.

Methods Employed:

Quantitative bacteriological techniques, such as bacteriological plate counts, quantitation of bacterial mass by turbidimetric methods; controlled use of known metabolic inhibitors to determine metabolic pathways.

Major Findings:

(A) The effect of inhibitors on bacterial L forms.

(1) Phenylethyl alcohol (PEA). This compound lyses the L forms of Streptococcus faecium F24. The PEA does not disaggregate the cell membrane as sodium dodecyl sulfate (SDS) does. (Collaborator: Mr. Thomas)

(2) para-fluorophenylalanine (FPA). This compound prevents the growth but does not kill the L form of Streptococcus faecium F24. The parent strain showed little inhibition by FPA. This differential activity was used to determine the precise time at which a parent strain protoplast becomes committed to growth in the L state. Other workers have shown that a protoplast morphologically shifts to the L state two hours after planting in a suitable growth medium. Physiological commitment, as determined with FPA, occurs 60-90 minutes after planting the protoplast. No L colonies developed when either protoplasts or stable L forms of F24 were plated on medium containing FPA. The L forms of other bacteria were tested for the inhibition by FPA. The L forms of gram-positive cocci (Streptococcus and Staphylococcus) are susceptible to 0.02 M FPA, while L forms of Corynebacterium and Pseudomonas were not inhibited by 0.04 M FPA. The strain of Pseudomonas L forms was unstable and reverted in the presence of FPA. The studies above reveal that a few L forms are sensitive to FPA, and FPA can be used to determine the point of physiological transition between the bacterial phase and the L phase.

(B) Previously, I reported that two strains of streptococcal L forms were unaffected by a variety of centrifugal conditions. Those studies were extended to representative organisms from other genera, i.e., Staphylococcus, Proteus, and Pseudomonas. The viability of suspensions remained the same after centrifugation and resuspension. Therefore, the viability of samples of a variety of L forms is unaffected by centrifugation. (Collaborator: Dr. McGee)

(C) Last year, the resistance of Group A streptococci to penicillin in the presence of 3% salt, was shown. There was no growth in 3% salt alone, but an additional possibility was the inactivation of penicillin by salt. This year, this latter possibility was ruled out by experiments showing the killing of enterococci by penicillin in all concentrations of salt up to 8%--concentrations in which the enterococcus grows well.

These results indicated that, solely because of absence of growth and cell wall synthesis of Group A streptococci in 3% salt, penicillin was ineffective; and suggested that the apparent reversion of L forms, carried in penicillin-containing media after induction by penicillin might, in fact, be due to streptococci carried over in inocula and unaffected by the penicillin in the L form media. This hypothesis was tested in a sham system (representing conditions of such culture of the L forms) in penicillin plus 3% salt. Group A streptococci were planted on the surface of the medium, and agar blocks were subcultured onto fresh medium after three days at 37 C, by the method used

for L forms. After two such subcultures, Group A streptococci were recovered from the final subculture by placing agar blocks into broth containing penicillinase. These results show that, under these conditions, false "reversion" of L forms might be found; and indicate the need for stringent controls.

Significance to Bio-Medical Research and the Program of the Institute:

If the presumptive pathogenicity of L forms is to be successfully investigated, it is important to define conditions which allow definition of the presence and state of cell wall-defective forms which may be isolated from clinical materials. These results on several basic properties define the influence of centrifugation, metabolic inhibitors, and salt on procedures commonly used. They indicate, for example, that centrifugation may be employed without detriment; and that FPA may be useful in differentiating an isolate as an L form (which it inhibits) from protoplasts, whole cells, or presumptively other stages of cell wall deficiency (which it does not inhibit).

Proposed Course:

As time permits, a series of selective media will be developed to differentiate L form inocula from other cell wall defective variants, and thus hopefully permit clarification of the pathogenicity of L forms of bacteria.

Honors and Awards:

None

Publications:

None

Serial No. NIAID 65H-1
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the fate of Group A streptococci phagocytized by human monocytes and polymorphonuclear leukocytes.

Previous Serial Number: Same

Principal Investigators: Dr. Alan D. Glick

Other Investigators: Dr. Jon M. Ranhand and Dr. Roger M. Cole

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | - | - | - | - |
| Professional | - | - | - | - |
| Other | - | - | - | - |

Project Description:

See 1972 Annual Report.

Project has been terminated.

Serial No. NIAID 77-1-F
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on *Aspergillus*

Previous Serial Number: Same

Principal Investigator: Kyung Joo Kwon-Chung

Other Investigators: Sang Jae Kim

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 24/12 | - | - | 24/12 |
| Professional | 16/12 | - | - | 16/12 |
| Other | 8/12 | - | - | 8/12 |

Project Description:

Objectives:

A new heterothallic species of *Aspergillus*, producing the conidial stage of the *Aspergillus fumigatus* series was isolated from the eyeballs of a rabbit. Objectives of this project are 1) to describe the new species, 2) to determine the genetic control of the mating system and biosynthesis of conidial pigment, and 3) to isolate mutant strains resistant to various polyene antifungal antibiotics.

Methods Employed:

Six original isolates of the new *Aspergillus* were crossed in all possible combinations on malt extract agar. The cleistothecia produced by a selected pair (AF 4 x AF 5) were dissected, and single ascospores were isolated. Two hundred and thirty-nine $F_1$ strains isolated were back crossed. Conidial suspensions of AF 4 and AF 5 were subjected to U.V. irradiation and plated out on malt extract agar to isolate the prototrophic-color mutants. The various conidial color mutants of opposite mating types were crossed, and the epistatic vs.

hypostatic relationship between the different pigment was analysed by testing the $F_1$ progeny. The cultures of AF 4 and AF 5 were subjected to U. V. irradiation or cultured successively on media containing polyene antibiotics of various concentrations to isolate the strains resistant to the drugs.

Major Findings:

The new species of *Aspergillus* produces a conidial stage closely resembling that of *A. fumigatus* and is the only heterothallic species among the members of *A. fumigatus* group. They occur in two mating types, A and a, and the incompatibility is controlled by 1-locus, 2-allele system. The optimum temperature for the growth is 37° C, and it survives in animal tissues for longer than six weeks without invading the tissues. Heterokaryon formation was not observed between the strains of our new species and other members of *A. fumigatus* group.

The hypostatic vs. epistatic relationship between the conidial color mutants showed that the biosynthesis of green wild-type spore color proceeds from white - → yellow - → beige - → brown - → green. The frequency of recombinants found among the $F_1$ progeny obtained from the crosses of the mutants was less than 3%, indicating a linkage of the genes controlling the pigment. The minimum inhibition concentration (MIC) of Amphotericin B for the wild type is 0.5 ug/ml. Sixteen strains resistant to the drug concentration up to 10 ug/ml were obtained by U. V. irradiation and continuous subculturing for twenty-three generations on the drug-containing media. The Amphotericin B resistant strains lose their ability of sexual reproduction. When the resistant strains are paired with the wild type of the opposite mating type, however, they can fertilize the opposite strains.

Significance to Biomedical Research and the Program of the Institute:

The single most medically important species in the genus *Aspergillus* is *A. fumigatus*. Genetic research with this fungus has not been done due to the lack of proper biological tools. Since our new species is heterothallic and closely related to *A. fumigatus*, the genetic knowledge resulted from this project may be applicable to *A. fumigatus*. Studies of the acquisition of resistance to Amphotericin B, the only drug effective in the treatment of aspergillosis, are of clinical importance.

Proposed Course of Project:

The project will continue to investigate 1) the genetic control on polyene drug resistance, 2) the difference in the fine structure of mycelium between drug-sensitive and drug-resistant strains, and 3) the change in sterol of the mycelium of the strains resistant to different kind of polyene antibiotics.

Honors and Awards:

None

Publications:

None

Serial No. NIAID 77-1-G
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Genetic study of pathogenic fungi

Previous Serial Number: Same

Principal Investigator: Kyung Joo Kwon-Chung

Other Investigators: None

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 28/12 | - | - | 28/12 |
| Professional | 8/12 | - | - | 8/12 |
| Other | 20/12 | - | - | 20/12 |

Project Description:

Objectives:

To investigate the distribution of the two mating types of *Histoplasma capsulatum* among soil isolates and clinical isolates from endemic areas of the United States.

Methods Employed:

A) One hundred eighty-two strains isolated from human cases of histoplasmosis and 939 isolates obtained from soil (by mouse passage technique) collected from 13 endemic states in the United States were tested for their mating types by crossing with + and - tester strains on Alphacel-yeast extract agar.

B) The mycelial form of all the isolates were inoculated on Blood-glucose-cysteine (BGC) agar and inoculated at 37° C to convert them into the yeast form.

C) A total of 20 soil isolates, 10 of + and 10 of - type, were randomly selected and injected into mice intravenously, both in yeast form and mycelial form to detect the difference in their virulence.

D) Various sets of parental strains of different phenotypes were crossed and $F_1$ progeny analysed.

Major Findings:

A) The results show that + and - strains of *H. capsulatum* exist in soil in an equal frequency. The exceptions to these results were seen among the isolates from Washington, D. C. and Maryland where only one mating type, either + or -, respectively, was isolated from each. In both states all these isolates came from the soil collected in limited foci over the period of time, compared to other states where soil collections were made from large areas at one time. This suggests that all 23 Maryland isolates and 11 D. C. isolates may have been originated from each single strain. Among the clinical isolates, however, the - type was 7.5 times more frequent than the + type, indicating that either the - type is more virulent or gains access to the human body more readily than the + type. (See Table 1.)

B) Twenty-two percent of the human isolates converted into the smooth yeast form on BGC agar at 37°, and they were all of - strains. Only 5% of the soil isolates converted to yeast form *in vitro*, and the ratio of + and - type among the converted strains was 1 (+) : 3 (-).

C) The results of experimental infections produced in mice by intravenous injection of yeast cells or mycelial phase showed that there is no significant difference in virulence between + and - strains.

D) Analysis of $F_1$ progeny obtained from the various crosses with a different phenotype showed the quantitative inheritance in the ability to form macroconidia, microconidia, and aerial hyphae.

Significance to Biomedical Research and the Program of the Institute:

The study on the distribution of + and - strains of *Histoplasma capsulatum* in soil, as well as in cases of human infection, shows that the - strain is either more virulent or gains access to human body more readily than the + type. Tests with all the mycelial forms *in vitro* suggest that the - type converts to tissue form (yeast cells) more readily than the + type.

Experimental infection in mice by intravenous injection of + and - type failed to show a difference in the virulence. Further study, using a more natural system such as exposing mice to an aerosol of spores, may offer an answer to the epidemiological significance of the - type.

Proposed Course of Project:

The project will be pursued to investigate 1) the genetic control of dimorphism, virulence and serotype of *H. capsulatum* and 2) the genetic relationship between *H. capsulatum* and *H. duboisii*.

Table 1:

| Geographic Origin | | No. of Strains | Mating Type + | - | ? | P-Value on the Estimated 1:1 |
|---|---|---|---|---|---|---|
| Arkansas | soil | 105 | 54 | 51 | | >0.9 |
| | human | 8 | 0 | 4 | 4 | - |
| Illinois | soil | 100 | 49 | 51 | | >0.9 |
| | human | 13 | 2 | 10 | 1 | 0.02 |
| Indiana | soil | 30 | 13 | 17 | | 0.6 |
| | human | 4 | 0 | 4 | | 0.04 |
| Iowa | soil | 17 | 7 | 10 | | 0.65 |
| | human | 0 | 0 | 0 | | - |
| Kansas | soil | 41 | 24 | 17 | | 0.42 |
| | human | 2 | 1 | 0 | 1 | - |
| Kentucky | soil | 3 | 1 | 2 | | - |
| | human | 30 | 7 | 22 | 1 | 0.005 |
| Maryland | soil | 23 | 0 | 23 | | <0.001 |
| | human | 1 | 0 | 1 | | - |
| Missouri | soil | 578 | 273 | 300 | 5 | 0.3 |
| | human | 86 | 8 | 76 | 2 | >0.0001 |
| Ohio | soil | 19 | 9 | 10 | | >0.9 |
| | human | 21 | 0 | 20 | 1 | <0.0001 |
| S. Carolina | soil | 2 | 0 | 2 | | - |
| | human | 6 | 2 | 4 | | 0.36 |
| Tennessee | soil | 1 | 0 | 1 | | - |
| | human | 3 | 1 | 2 | | - |
| Texas | soil | 9 | 5 | 4 | | >0.9 |
| | human | 6 | 0 | 6 | | 0.014 |
| Virginia | soil | 0 | 0 | 0 | | - |
| | human | 1 | 0 | 1 | | - |
| Washington D.C. | soil | 11 | 11 | 0 | | 0.0009 |
| | human | 0 | 0 | 0 | | - |
| Total | soil | 939 | 446 | 488 | 5 | 0.18 |
| | human | 182 | 21 | 151 | 10 | <0.0001 |

Honors and Awards:

The investigator is invited to serve as author of an article, "Genetics of pathogenic fungi," in the book, Critical Review of Microbiology, by CRC press, 1973.

Publications:

Kwon-Chung, K. J.: *Emmonsiella capsulata:* perfect state of *Histoplasma capsulatum*. Science 177: 368-369, 1972.

Kwon-Chung, K. J.: Studies on *Emmonsiella capsulata* I. heterothallism and development of the ascocarp. Mycologia 65: 109-121, 1973.

Glick, A. D. and Kwon-Chung, K. J.: Ultrastructural comparison of coils and ascospores of *Emmonsiella capsulata* and *Ajellomyces dermatitidis*. Mycologia 65: 216-220, 1973.

Wong, V. G., Kwon-Chung, K. J., Green, W. R., Anderson, R. R., Collins, E. M., and Hill, W. B.: Focal choroidopathy in experimental ocular histoplasmosis. Transaction of the American Academy of Opthalmology and Otolaryngology. In Press.

Young, N. A., Kwon-Chung, K. J., and Freeman, J.: Subcutaneous abscess caused by Phoma sp. resembling *Pyrenochaeta romeroi:* Unique fungal infection occurring in immunosuppressed recipient of renal allograft. Am. J. Clin. Pathol. In Press.

Serial No. NIAID 79-B-1
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Host Response to Pathogenic Fungi in Neoplastic Disease and Immunosuppression

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: Michael Worthington (L.V.D.)

Cooperating Units: Laboratory of Viral Diseases; Hematology and Supportive Care Branch, and the Baltimore Cancer Research Center, N.C.I.

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 7/12 | - | - | 7/12 |
| Professional | 2/12 | - | - | 2/12 |
| Other | 5/12 | - | - | 5/12 |

Project Description:

Objectives:

A. To study the antibody response to fungal infection in leukemic patients, and its use in laboratory diagnosis.

B. To determine the effect of immunosuppressive or cancer chemotherapeutic drugs on experimental fungal infection in mice.

Methods Employed:

A. Serum samples are tested for antibodies to antigens of *Candida albicans* antigenic group A. Heat-killed whole yeast cells are used as antigen for agglutination, a soluble extract (S) from sonically disrupted viable yeast cells, and mannan (M) from yeast cells are used as antigens in immunodiffusion. For agglutination, a four-fold rise in titer is interpreted as a positive

response. The observation of a precipitin band with either soluble antigen is interpreted as a positive reaction.

B. The effects of neonatal thymectomy, or the administration of antithymocyte serum (ATS) or cyclophosphamide (cytoxan) on laboratory infections with *Coccidioides immitis* and *Histoplasma capsulatum* in mice were tested.

Mice that had been thymectomized within 24 hours after birth were obtained commercially. Sham operated mice but with thymus left intact were used as controls. Cyclophosphamide (150 mg/kg body weight intraperitoneally) was injected on day 1, and 100 mg/kg body weight on days 8 and 15 after injection with the respective pathogen. Twenty-five hundredths of a cc of ATS was given intraperitoneally 1 day before infection and 1, 3, 7 and 11 days after. Normal serum was given to control groups. Death was used as the endpoint and the percent mortality calculated.

Major Findings:

A. Two hundred and four serum samples from 107 leukemic patients, with or without known Candida infection, were tested for antibodies to Candida. Many of these were single samples and intended to determine a baseline for future reference. In 8 patients an antibody rise to Candida was shown by agglutination and immunodiffusion. Tests on 9 other patients indicated that they had an agglutination titer of 1:160 or more and precipitating antibodies. For this latter group of patients only a single serum specimen was submitted for testing, or if multiple samples were submitted, all had elevated titers. Clinical evidence of candidiasis was present in 3 patients that showed an antibody rise, and in 4 of those that had elevated levels. In the other patients candidiasis was suspected but not proven culturally.

B. Mice immunosuppressed by neonatal thymectomy, ATS, or cyclophosphamide were markedly more susceptible to coccidioidomycosis and histoplasmosis than non-immunosuppressed mice. Immunosuppressed mice infected with *H. capsulatum* died before circulating antibody could be detected in the control animals. Injection of pooled anti *H. capsulatum* serum from non-suppressed mice did not protect or extend the life of cyclophosphamide or ATS-treated mice that were infected with *H. capsulatum*. The results suggest that cellular immune processes are more important in recovery from these infections than is the formation of humoral antibody.

Significance to Biomedical Research and the Program of the Institute:

Fungal diseases are serious complications of chemotherapy that affects cellular immunity. The studies reported herein clearly show that the course of either coccidioidomycosis or histoplasmosis, both of which may cause serious primary disease, is enhanced by thymectomy, the administration of antithymocyte serum, or cyclophosphamide. It is apparent that patients who are immunosuppressed should avoid exposure to these fungi. Invasive or deep-seated candidiasis is often difficult to diagnose in leukemic patients. Serology appears to offer an additional laboratory procedure to aid in that diagnosis.

Proposed Course of Project:

To continue to collect more information concerning the interpretation and limitations of Candida serology and to exchange reagents with other laboratories doing this type of testing to achieve standardization of methods and reagents.

Honors and Awards:

Chairman, Standards and Examining Committee for Certification in Medical Mycology, American Board of Medical Microbiology

Publications:

Preisler, H. D., Hasenclever, H. F., Henderson, E. S., Smith, J. K., and Louria, D. B.: A prospective study of anti-cancer interfering factor in leukemic patients. Cancer 30: 294-299, 1972.

Worthington, M., Rabson, A. S., and Hasenclever, H. F.: Effect of immunosuppression on experimental fungal infections. In Press.

Serial No. NIAID 79-C-1
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Ecologic and Aerobiologic Studies of *Histoplasma capsulatum*

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: K. J. Kwon-Chung

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 24/12 | - | - | 24/12 |
| Professional | 3/12 | - | - | 3/12 |
| Other | 21/12 | - | - | 21/12 |

Project Description:

Objectives:

To study the factors that contribute to the presence or absence of *Histoplasma capsulatum* in the soil.

Methods Employed:

The sites under study, where *H. capsulatum* is known to have been present for years, are a former bat roost at Clarksburg, Maryland, and at 7th Street and Pennsylvania Avenue, Washington, D. C., a former starling roost. Soil samples are collected at intervals and the absence or presence of the fungus is determined by mouse inoculation. The growth and survival of *H. capsulatum* in soil cultures in the laboratory are measured by direct cultural methods and mouse inoculation.

Major Findings:

Five of eighty soil samples collected in the spring and fall at 7th Street and Pennsylvania Avenue were positive for *H. capsulatum*. Two of the positive

samples were taken at the same location within the site. The other positives were from areas that had been negative for the past year but had been positive in previous years. Although this site has not been an active roost for starlings for ten years, and possibly diminishing in quantity, the fungus can still be isolated. At Clarksburg the addition of bat guano to a specific area (1 x 2 meters) within that site has not affected the presence of *H. capsulatum*. This study has been in progress for three years. Of twelve soil samples collected immediately before the first application of guano, one was positive for the fungus. Subsequent sampling at approximately six-month intervals have given the following results: (positives/number samples) 0/6, 1/12, 0/21, 0/12, 1/30, 1/20, and 1/20. A total of 1.5 kg of sterile guano was applied at six different times during the period of testing. At other locations in this site *H. capsulatum* can be isolated as in previous years. The house that harbored the bats was destroyed seven years ago.

Studies to determine the accuracy of the mouse injection technique for the isolation of *H. capsulatum* from soil have been done. Using sterile soil as a culture medium for the fungus and then allowing dessication of the medium at room temperature, a remarkably accurate comparison has been possible. Parallel inoculation of pulverized soil from the culture, using the proper serial dilutions, onto agar plates or injection into mice, indicate that a single viable unit will either give rise to a single colony *in vitro* or infect a mouse. Under these ideal conditions the method is highly sensitive, but for use with field samples many unknown factors are introduced. This work does permit greater confidence and reliance in the technique.

Significance to Biomedical Research and the Program of the Institute:

The frequent association of *H. capsulatum* with urban or rural avian and chiropteran roosts constitutes a public health problem. Information on the longevity and factors contributing to the existence of the fungus in these areas is being contributed by this project. This may permit the development of a rational biological control of the fungus in its natural habitat.

Proposed Course of Project:

To emphasize the effect of other additives that may affect the presence of *H. capsulatum* in an established site and its dispersal into air.

Honors and Awards:

Aerobiology Advisory Committee, United States National Committee, International Biological Program, H. F. Hasenclever

Publications:

Hasenclever, H. F.: Histoplasmosis in bats. Health Laboratory Science 9: 125-132, 1972.

Hasenclever, H. F., and Piggott, W. R.: Air sampling for *Histoplasma capsulatum*. U.S./IBP Aerobiology Program Handbook Number 3, Proceedings of Workshop/Conference II, 1972, pp 159-162.

Serial No. NIAID 79-D
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunochemical Studies on Polysaccharides from Fungi

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: Errol Reiss

Cooperating Units: None

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 33/12 | - | - | 33/12 |
| Professional | 15/12 | - | - | 15/12 |
| Other | 18/12 | - | - | 18/12 |

Project Description:

Objectives:

To study (1) antigenic relationships within or among species of yeasts; and (2) the molecular structure, serologic properties, and the haptenic groups of polysaccharides or other antigens isolated from yeasts and fungi.

Methods Employed:

The serologic reactive substances are extracted by carefully controlled methods from fungal cells or isolated from the culture media in which the fungi were grown. Serologic similarity or diversity are shown by agglutination, immunodiffusion, or quantitative precipitation. Biologic activity is shown by injection into hypersensitive animals. The monosaccharide content of polysaccharides is determined by gas chromatography. Antisera are prepared by the injection of rabbits with fungal cells.

Major Findings:

The three serotypes of *Saccharomyces telluris* have been described as A, B, and C. Serotype A has the broadest antigenic structure and is represented by a single reference strain. Serotype B has been thus far shown to be associated only with *Columba livia*, the common pigeon, and two reference strains. Serotype C appears to have the most limited antigenic structure of the three and has been isolated from a variety of columbid species and is also represented by two reference strains. An antigenic classification of the serotypes could be as follows: Serotype C - antigen 1, Serotype B - antigens 1 and 2, and Serotype A - antigens 1, 2, and 3. These serologic characteristics can be shown by agglutination of whole yeast cells with reciprocally absorbed antisera or by immunodiffusion tests using extracts of whole cells. The principal, if not the only, serologically active ingredients of the extracts of all the serotypes are mannans.

Quantitative procedures have shown that the total extractable materials of antigenic Groups A and B of *Candida albicans* are of the same magnitude. When these extracts are compared with mannans using cellulose acetate electrophoresis and staining with periodic acid Schiff reagent, at least one addition component may be seen. This substance may be cell wall glucan. The only detectable difference between the two groups, however, remains their serological activity, and this has been shown previously by investigations of this project to be due to differences in the structural characteristics of their mannans.

A serologically active polysaccharide has been isolated from the mycelium of *Histoplasma capsulatum*. This substance has been characterized by gas chromatography as containing mannose and galactose at a ratio of 2.5: 1, respectively. It will precipitate antisera to the fungus and elicit a delayed reaction in infected guinea pigs.

Significance to Biomedical Research and the Program of the Institute:

The results show that serotypes have been identified in another species of yeast, and that the mannans found on the surface of the yeast cell determine their serologic character. More knowledge of the antigenically active polysaccharides of species of Candida and other pathogenic fungi will elucidate some problems and aid in the interpretation of serologic reactions used in the diagnosis of fungal disease. These studies are defining the antigens that provoke antibody responses and induce delayed hypersensitivity in some fungal infections.

Proposed Course of Project:

To complete the investigations already in progress comparing the serologic reactivity of the *S. telluris* serotypes with *C. albicans* Groups A and B, *Torulopsis glabrata*, and *Saccharomyces cereviseae*. To continue to isolate and characterize polysaccharides and glycopeptides from the mycelial and yeast forms of *H. capsulatum*, and *C. albicans*, A and B.

Honors and Awards:

None

Publications:

Hasenclever, H. F., and Kocan, R. M.: Serotypes in *Saccharomyces telluris:* Their relation to source of isolation. Infection and Immunity, 7: 610-613, 1973.

Serial No. NIAID 79-E
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Yeast Flora of Birds

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: None

Cooperating Units: Patuxent Wildlife Research Center, Dr. R. M. Kocan

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 4/12 | - | - | 4/12 |
| Professional | 4/12 | - | - | 4/12 |
| Other | - | - | - | - |

**Project Description:**

Objectives:

To study qualitatively the yeast flora of columbiform birds with particular emphasis on *Columba livia* (common pigeon) and determining the frequency of isolation of those microorganisms that are associated with and cause disease in humans.

Methods Employed:

The throats, crops, or cloaca of live or recently sacrificed birds are swabbed, and the swabs are used to inoculate glucose-neopeptone agar plates. The plates are incubated at 37° C, and after 48 hours all the different colonial types of yeast are picked and subcultured on malt extract agar slants. The yeasts are then identified according to the morphological and physiological characteristics given in The Yeasts--a taxonomic study, J. Lodder (Ed.), (2nd. edition), North Amsterdam Publishing Company, 1970.

Major Findings:

Yeasts were isolated from seven species of pigeons and doves studied. The frequency of isolation ranged from 95% to 14%. *Candida albicans* was the most common organism isolated. *Saccharomyces telluris* continues to be the next most commonly isolated yeast. All the strains of *S. telluris* isolated from *C. livia* are of a single serotype, whereas all those obtained from the other avian species belong to another serotype.

Studies designed to show variations in the frequency of isolations among groups of feral pigeons collected monthly over one year indicated that for the three most common yeasts isolated, the range for *C. albicans* was 23 - 88% (average 59%), *S. telluris* 3 - 96% (average 50%), and *C. krusei* 0 - 95% (average 32%). One group of birds, when confined to outdoor cages and cultured at monthly intervals for three months, gave these results: *C. albicans* - 75%, 70%, and 63%, respectively, *S. telluris* - 55%, 23%, and 6%, and *C. krusei* - 60%, 0%, and 6%. It appears that *C. albicans* is a stable member of the oral and gastrointestinal biota of these birds.

Significance to Biomedical Research and the Program of the Institute:

These results extend our knowledge concerning the ecological relationship for some of the yeasts, particularly members of the genus Candida, that are pathogenic to man and animals. The common pigeon is a close associate of man and may represent a source of contagion for these yeasts. The significance of the isolation of a single serotype of *S. telluris* from pigeons, and a different serotype from other species of columbiforms, remains to be determined.

Proposed Course of Project:

To continue testing for monthly variations in the species of yeasts isolated, to culture birds collected from other geographical locations, and to determine the virulence of *C. albicans* for pigeons.

Honors and Awards:

None

Publications:

Kocan, R. M. and Hasenclever, H. F.: Normal yeast flora of the upper digestive tract of some wild columbids. Journal of Wildlife Diseases 8: 365-368, 1972.

Serial No. NIAID-207
1. Microbiology
2. Mycoplasma
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Basic Studies on Mycoplasma

Previous Serial Number: Same

Principal Investigator: Dr. Joseph G. Tully

Other Investigators: Dr. Roger M. Cole

Cooperating Units: Dr. J. M. Bové, Centre de Recherche Bordeaux, France
Dr. R. F. Whitcomb, Plant Industry Station, USDA, Beltsville, Maryland

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 48/12 | - | - | 48/12 |
| Professional | 12/12 | - | - | 12/12 |
| Other | 36/12 | - | - | 36/12 |

Project Description:

Objectives:

The objectives of these studies cover both basic and applied aspects of mycoplasmas and related wall-free procaryotes, including their nutrition and metabolism, mechanisms of virulence, immunological relationships among human and animal strains, and their role in human infection and certain other mixed bacterial, viral and protozoan diseases of man and animals.

Methods Employed:

Recovery of mycoplasmas and related organisms from various hosts was accomplished by standard cultivation techniques and all agents were serologically typed by fluorescent antibody or metabolic-inhibition procedures. Characterization of sterol-nonrequiring mycoplasmas and spiroplasmas was made on the basis of biochemical and serological properties and on cell protein

patterns obtained by gel electrophoresis. Animal mycoplasmas were compared in a variety of biological and serological procedures to other prototype human and animal mycoplasmas in the current culture collection. The gel electrophoretic pattern of wall-free procaryotes proteins was also used to confirm the serological relationships among various strains.

Major Findings:

A. Continued study of the sterol-nonrequiring mycoplasmas (acholeplasmas) has provided new and substantial evidence of the widespread distribution of these organisms in a variety of hosts while at the same time presenting information on their fundamental differences from the sterol-requiring mycoplasmas. On the basis of extensive biological and serological tests we have defined at least two new groups (Squire and 19 L) of *Acholeplasma* species and provided additional means of characterizing the three established species in this genus. The biological properties found most useful in classifying the individual species and groups included carbohydrate fermentation patterns, production of pigmented carotenoids, hydrolysis of aesculin, and the polyacrylamide gel electrophoresis patterns of cell proteins. Serologic analysis was confined to the growth-precipitation and growth-inhibition techniques, since a very detailed study of the application of the metabolic-inhibition test to these organisms gave unsatisfactory separation of the individual species. The two new groups of acholeplasmas outlined here are of special significance since representatives of each have been implicated as tissue culture contaminants and both groups are unrelated to established *Acholeplasma* species. The Squire group has been traced to bovine hosts. Group 19 L appears to be predominately of caprine origin as two strains in this group were recovered from the eyes of goats with keratoconjunctivitis. Three other strains in this group were not identified as to host origin but all were recovered from cell cultures. Since few mycoplasmas of goats have been found as tissue culture contaminants, it is thought that this group of acholeplasmas might also occur in bovines--the most frequent source of cell culture contaminants. In addition to these two new groups, five other individual acholeplasmas are under examination as possible new types.

B. In a collaborative study with Dr. J. M. Bové and Dr. R. M. Cole, we have characterized the first mycoplasma-like organism of plant origin. The organism recovered from "stubborn" disease of citrus was obtained in pure culture and the morphological, biological, and serological properties of two isolates (California and Morocco) established. Organisms plated to solid medium produced typical mycoplasma "fried egg" colonies under both aerobic and anaerobic environments. Detailed tests on their sterol requirements indicated that serum or cholesterol was required for growth. Optimum temperature for growth was 32 C. Significant numbers of the organism passed through 220 nm membrane filters. Biochemical tests indicated that the organisms fermented glucose and possessed most of the biochemical properties of mycoplasmas. The organisms were resistant to penicillin but sensitive to tetracycline, amphotericin B and several other antibiotics. The acrylamide gel electrophoretic patterns of the two strains were identical but distinct from other acholeplasmas and mycoplasmas. Examination of the DNA properties of the two strains indicated that they possessed a G+C content of 26 mole % and had a genome size of approximately 1 X $10^9$ daltons--properties that

are comparable to other mycoplasmas. Extensive analysis of the serological properties of the citrus organism against antisera to over 50 other mycoplasmas showed it to be distinct from all other described mycoplasmas. Broth cultures of the organism received in the Section were first examined under phase contrast and darkfield microscopy. This was the first evidence that the basic structure of this organism consisted of helical forms. Additional study revealed that the motile helical forms occurred most frequently during the active growth phase of the culture. Ultrastructural studies confirmed the helical morphology of the organisms, the absence of a true cell wall or other structures present in spirochetes, and that both strains were infected with a tailed bacteriophage. A proposal to classify the new mycoplasma-like agent from plants as *Spiroplasma citri* has been made.

C. The characterization of spiroplasmas as a new mycoplasma-like organism opened the way to study the relationship of these organisms to other wall-free procaryotes. The most likely candidate for comparison was the helical mycoplasma-like organism observed in, but not cultured from, corn stunt disease. Earlier studies on the citrus mycoplasmas (*Spiroplasma citri*) suggested that techniques employed in the serological analysis of conventional mycoplasmas might be applied to this comparison. Growth-inhibition and precipitin tests, using antisera prepared against plant material from corn stunt disease, indicated that *S. citri* and the corn stunt agent were serologically related--but perhaps not identical. Adsorption of anti-corn stunt serum with *S. citri* removed the reacting antibody while adsorption with other mycoplasmas failed to alter the serological reactivity. Additional confirmation of this relationship was obtained by demonstrating that antiserum to healthy corn material did not react with *S. citri* or to other mycoplasma antigens. These findings, in collaboration with Dr. R. F. Whitcomb, also led to a study of the interaction of mycoplasmas and spiroplasmas in the insect vectors of some suspected spiroplasma plant diseases. *Acholeplasma laidlawii* and *A. granularum* both multiplied to over 3 $\log_{10}$ units from the amount initially injected into individual leafhoppers. *Spiroplasma citri* not only multiplied to the same extent in all three insect species tested, but also decreased the longevity of the insects. *Mycoplasma hominis* injected into insects failed to show significant growth but persisted for at least eight days. These observations suggest that a novel group of organisms (spiroplasmas), with greater similarity to mycoplasmas than any other presently recognized group of wall-free procaryotes, exist as pathogens.

D. Additional studies have been carried out on several groups of human and animal mycoplasmas in attempts to characterize their biological relationships. One group, representing four goat mycoplasmas and two isolates of human origin, possesses similar properties. Confirmation of this association is being established through an analysis of their cell protein patterns. This group and two additional goat mycoplasmas (Goat 145 and KS-1) appear to be unrelated to all other human and animal mycoplasmas. The initial association of these strains with animal diseases (arthritis and purulent joint infections) provides new agents to examine factors responsible for mycoplasma virulence.

Significance to Bio-Medical Research and the Program of the Institute:

Our major effort has been directed toward a clarification of the relationships among various acholeplasmas, mycoplasmas, and other wall-free procaryotes in an effort to assess their basic biological and serological characteristics. With this information it will be possible to determine more adequately the role of these organisms in human disease and in diseases of undetermined etiology. Studies on the acholeplasmas and spiroplasmas have supplied, and will continue to supply, useful insights into an understanding of general membrane structure and function. The spiroplasmas will be especially useful tools to determine membrane organization and how an organism maintains a helical structure in the apparent absence of cell wall material. Infection of spiroplasmas with a tailed bacteriophage, and the previously noted infection of acholeplasmas with filamentous viruses, again raise questions about the mechanism of virus attachment to membranes and what role viruses play in virulence of these organisms. Thus, the findings reported here enlarge our concepts of the nature and distribution of wall-free procaryotes and present their advantages as biological tools.

Proposed Course of the Project:

Further studies are being directed to specific animal mycoplasmas that might offer appropriate models for establishing the role of these agents in human disease. Techniques are being developed that will enhance the recovery of latent or non-culturable mycoplasmas from both cell culture systems and the intact animal host, in hopes that these methods can be applied to human disease problems. Finally, additional effort is being directed to a more complete understanding of mycoplasma neurotoxin and other virulence factors in mycoplasmas and other wall-free organisms.

Honors and Awards:

Chairman and Member, WHO/FAO Board of Animal Mycoplasma Characterization

Member, International Subcommittee on Taxonomy of the Mycoplasmatales

Member, Committee on Civil Service Standards, American Academy of Microbiology

Chairman, Organizing Committee for International Symposium on Mycoplasmas of Human, Animal, and Plant Diseases, Bordeaux, France, September 1974

Invited participant in seminar on "Diagnostic Mycoplasmology of Isolates from Animals and Man" and presented paper on "Isolation and Identification of Mycoplasmas from Sheep and Goats" at 73rd Annual Meeting of the American Society for Microbiology, Miami Beach, Florida, May 6-11, 1973. Also presented paper in Mycoplasma Session on "Multiplication of mycoplasmas and mycoplasma-like organisms in leafhoppers."

Chaired session on "Mycoplasmatales - Newer Agents" and presented paper on "Biological and serological characteristics of the acholeplasmas" at New York Academy of Sciences Conference on "Mycoplasmas of Human, Animal, and Plant Diseases, January 8-10, 1973

Publications:

Tully, J. G. and Mercado, T. I.: Mixed infections: the interaction of mycoplasmas and malarial parasites. In K. Elliott and J. Birch (eds), Ciba Foundation Symposium on Pathogenic Mycoplasmas, Elsevier, Amsterdam, p. 285-300, 1972.

Edward, D. G. ff, Freundt, E. A., Chanock, R. M., Fabricant, J., Hayflick, L., Lemcke, R. M., Razin, S., Somerson, N. L., Tully, J. G., and Wittler, R. G.: Proposals for minimal standards for description ot new species of Mycoplasmatales, class Mollicutes. Int. J. Syst. Bacteriol., 22: 184-188, 1972.

Armstrong, D., Morton, V., Yu, B, Friedman, M. H., Steger, L. and Tully, J. G.: Canine pneumonia associated with Mycoplasma infection. Amer. J. Vet. Res., 33: 1471-1478, 1972.

Tully, J. G.: Biological and serological characteristics of the acholeplasmas. Ann. N.Y. Acad. Science, 1973,In Press.

Saglio, P., Lhospital, M., Lafleche, D., Dupont, G., Bové, J. M., Tully, J. G., and Freundt, E.A.: *Spiroplasma citri* gen. and sp. nov.: A new mycoplasma-like organism associated with "stubborn" disease of citrus Int. J. Syst. Bacteriol., 1973, In Press.

Cole, R. M., Tully, J. G., Popkin, T. J., and Bové, J. M.: Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma-like organism (*Spiroplasma citri* gen. and sp. nov.) from "stubborn" disease of citrus. J. Bacteriol., 1973, In Press,

Bové, J. M., Saglio, P., Tully, J. G., Freundt, E. A., Lund, J., Pillot, J., and Taylor-Robinson, D.: Characterization of the mycoplasma-like organism associated with stubborn disease of citrus. Ann. N.Y. Acad. Science, 1973, In Press.

Cole, R. M., Popkin, T. J., Tully, J. G., and Bové, J. M.: Ultrastructure of the agent of citrus "stubborn" disease. Ann. N.Y. Acad. Science, 1973, In Press.

Serial No. NIAID 152
1. Microbiology
2. Immunology
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on antibody production and mechanisms of hypersensitivity

Previous Serial Number: Same

Principal Investigator: Dr. Sanford H. Stone

Other Investigators: Julius H. Goode, Jr.

Cooperating Units:

1. Dr. Marius Valsamis
   Dr. Cedric Raine
   Division of Neuropathology
   Albert Einstein College of Medicine
   New York, New York

2. Dr. Gardner Middlebrook
   Dr. Zulema Reggiardo
   Division of Experimental Pathology
   University of Maryland School of Medicine
   Baltimore, Maryland

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 24/12 | - | - | 24/12 |
| Professional | 24/12 | - | - | 24/12 |
| Other | - | - | - | - |

Project Description:

Project Subtitle I: Mechanisms of protection against Mycobacterium tuberculosis (in collaboration with Cooperating Unit #2)

Objectives:

To elucidate the mechanisms underlying protection against tuberculosis in guinea pigs.

Methods Employed:

Inbred histocompatible guinea pigs were sensitized by inoculation of living BCG vaccine or injection of killed M. tuberculosis in oil emulsion. Lymph node or spleen cells were taken during various stages of the immune response and transferred to histocompatible and random bred recipients. Recipients were challenged by aerosols containing living M. tuberculosis Organs were examined for the presence of M. tuberculosis and its lesions by bacteriological and histological criteria.

Major Findings:

This subproject, in its early stages, is determining optimal conditions for the transfers described above. For example, it has been determined that delayed hypersensitivity to tuberculin is poorly transferred by spleen cells after systemic immunization with living BCG.

Significance to Bio-Medical Research and the Program of the Institute:

Although the search for better vaccines for tuberculosis has occupied the attention of investigators for many decades, the actual mechanisms of protection against this disease remain unexplained. The elucidation of the components of the immune response involved in protection would lead to better choices of form of antigen and/or adjuvant. Passively transferable mechanisms of importance in therapy of other diseases (tumors, leprosy) also require clarification.

Proposed Course of Project:

Lymph node cells, spleen cells, peritoneal exudate cells, and sub-groups of T and B cells from them will be adoptively transferred from BCG immunized histocompatible donors in attempts to determine the components of the immune response capable of protection against the disease.

Project Subtitle II: Genetics and pathogenesis of acute and chronic encephalomyelitis

Objectives:

To elucidate the mechanisms underlying resistance to autoimmune encephalomyelitis (AE) linked to age, sex, or strain differences in guinea pigs, in search for information on the etiology of the disease or of demyelinating diseases in general. (In collaboration with Cooperating Unit #1)

Methods Employed:

Young and mature, male and female strain 2 and strain 13 guinea pigs were sensitized with homologous spinal cord incorporated into Freund's complete adjuvant (FCA) containing killed Mycobacterium tuberculosis. The effects of subsequent active or possive immunization with the same antigen were observed. Tissues from guinea pigs with acute, sub-acute, and chronic AE were taken for detailed comparison of these conditions using classic histological methods and analyses by electron microscope (EM) as well.

Major Findings:

EM studies show that in guinea pigs resistant to the acute form of AE, the central nervous system has lesions of different ages showing various degrees of demyelination and several stages of remyelination. Thus in same animals, a chronic form of AE with multiple bouts of disease and recovery can be traced.

Significance to Bio-Medical Research and the Program of the Institute:

AE of guinea pigs is an acute disease which reduces its importance as a potential model for multiple sclerosis (MS) in man. Animals die before any demyelination occurs. The discovery of conditions which produce a more chronic disease, which shows patterns of demyelination and remyelination in several cycles, now provides an animal model for human multiple sclerosis. Furthermore, the autoimmune pathogenesis theory of MS is kept in contention by such a model, since AE is a proven autoimmune disease in rodents.

Proposed Course of Project:

Living lymphoid cells and their products from acute, sub-acute and chronic AE guinea pigs will be assayed in tissue cultures of myelinated CNS tissues and results correlated with the particular pathology of the varieties of AE.

Project Subtitle III: Studies on cellular manifestations of hypersensitivity and immunity in guinea pigs; transplantation of immunologically competent tissues

Objectives:

Correlation of the developments in lymphoid tissues draining the sites of injection of antigens with the capacity of the lymph node T or B cells to transfer either hypersensitivity or antibody-producing capacity.

Methods Employed:

Immune deviation (suppression of manifestation of delayed hypersensitivity) was established by injection of alum-precipitated antigens in guinea pigs. In appropriate passive versions of the model, guinea pigs were sensitized with various antigens incorporated into complete or incomplete Freund's adjuvant or in alum adjuvants. They were used as donors or recipients of lymphoid cells, in order to isolate and characterize the role of the adjuvant in selecting components of the immune response.

Major Findings:

Prior injection of alum-precipitated antigens will, during the induction of immune deviation of delayed type reactions, for example, suppression of cutaneous basophil hypersensitivity, produce enhancement of Arthus type

reactions in the guinea pig. Since $7SIgG_2$ antibodies are reduced during immune deviation, this questions their importance in the Arthus response of guinea pigs or indicates two phases in their production under these circumstances.

Significance to Bio-Medical Research and the Program of the Institute:

The types of cells required for transfer of adoptive immunity are of interest for the reconstruction of the immune response at the cellular level. Which cells are susceptible to immune deviation and also the mechanism of immune deviation may be queried by this approach. The adoptive transfer is a powerful tool for study of the cell-cell interactions recently recognized of great importance in immune responses. The importance of the initial adjuvant stimulation observed in deviation studies is of obvious significance to those in search of more efficient vaccines.

Proposed Course of Project:

Transfers involving cell suspensions of known composition and of slices of lymphoid tissue from sensitized deviated donors will be made into normal or deviated recipients to assess role of cell-cell association or of certain tissue architecture in normal or deviated immune responses.

Honors and Awards:

Professorial lecturer in immunology, Howard University, Washington, D.C.

Publications:

None

LABORATORY OF BIOLOGY OF VIRUSES

1973 Annual Report

Index


| | Page |
|---|---|
| Summary | 1 |
| Serial No. | |
| 80A - Biochemical Characterization of Nucleic Acids of Transformed and Virus Infected Cells | 9 |
| 80G - Characterization and Biosynthesis of Viral Enzymes and Structural Proteins | 13 |
| 80H - Studies on the Replication of the Parvovirus, KRV | 18 |
| 84B - Studies with Isolated Metaphase Chromosomes | 22 |
| 84C - Mechanism of DNA Replication and Integration. | 25 |
| 85A - Structure of SV40 DNA | 28 |
| 87A - Properties of the Nucleic Acid and Protein Components of Adenovirus-Associated Viruses | 31 |
| 87B - Properties of Adenovirus DNA | 35 |
| 88 - Studies on the Synthesis of Proteins in SV-40 Infected Cells | 38 |
| 88B - Molecular Biology of Chromatin and Regulation of Viral Gene Activity in Cell Transformation | 41 |

# LABORATORY OF BIOLOGY OF VIRUSES

The general area of research of this laboratory continues to be molecular virology. Members of the Laboratory of Biology of Viruses have used knowledge of molecular biology in order to gain an understanding as to how human and animal viruses affect cells and how they replicate. Studies on the mechanism of transformation of cells by oncogenic viruses continue as a major area of investigation. This past year has been highly productive and during this period many notable findings have been made.

## The Mechanism of Replication of SV40 DNA Has Been Further Defined

In previous studies we were able to show that DNA synthesis occurred at a specific site in the covalently closed duplex molecule. This finding has been confirmed by cleaving replicating SV40 DNA molecules with a bacterial restriction endonuclease. Examination of the cleaved molecules by electron microscopy has confirmed that replication is initiated at a specific site and further, has demonstrated that replication is bi-directional, that is there are two replicating forks that both move at the same rate in opposite directions. In a separate series of experiments the termination site for DNA replication has been located and is 50% of the genome length from the DNA initiation site. This finding is in complete agreement with bidirectional DNA replication.

A second series of experiments have defined the mechanism of chain growth during SV40 replication. Chain growth occurs by a discontinuous mechanism in which short 4S DNA fragments are made and then joined to growing DNA chains. These DNA fragments have been isolated, and they are able to reanneal to form duplex structures. This establishes that the two growing chains at each replication fork are made in a discontinuous manner. Inhibitor studies indicate that two DNA polymerases may be involved in chain growth. The first synthesizes the 4S fragment while the second must fill in a gap between the 4S fragment and the growing chain before it is joined by the action of ligase.

A third series of studies have been concerned with the mechanism of effecting semi-conservative replication of a covalently closed duplex. These studies have demonstrated (1) a decrease in the topological winding number which accompanies replication and, (2) evidence for a denatured region about twenty nucleotides in length at each of the replication forks.
(Salzman, Fareed, Sebring, Thoren)

## Chemical Modification of SV40 DNA

Several studies from different laboratories has led to a model for super-helical DNA (form I) in which there is a region or regions of altered secondary structure containing unpaired bases. In order to map these region(s) and test their biological role in SV40 DNA (form I), it is essential that we modify the unpaired bases with a radioactive reagent that is not readily reversible. Experiments using a water soluble carbodiimide reagent shows that form I reacts whereas nicked SV40 (form II) does not. The reaction is detected by sedimentation velocity and buoyant density

changes. These studies were performed with cold reagent and further characterization awaits the synthesis of $C^{14}$ labeled carbodiimide. Several other chemical modifications will also be tested (Lebowitz, Salzman).

Messenger RNA in Transformed and Lytically Infected Cells Has Been Characterized

It was previously shown that during lytic infection of green monkey cells, 30-35% of the minus DNA strand and 65-70% of the plus DNA strand are expressed. Similar studies, carried out with 12 SV40 transformed cell lines, have indicated that transcription occurs predominantly from the minus strand of the integrated viral genome. In 10 of 12 lines examined 30-50% of the minus viral DNA strand is expressed; in 2 lines, 65-75% is transcribed. The portion of the minus DNA strand expressed in all of the transformed cell lines tested also contains the viral genetic information transcribed during the early phase of the lytic cycle. Transcription of the plus DNA strand was detected in only 3 of 12 lines; less than 10% was expressed in all cases.

In collaboration with Dr. Daniel Nathans it has been possible to topographically locate the "early" and "late" regions of SV40 DNA on the physical map of the viral genome. The "early" segment is included in DNA sequences corresponding to the minus strands of H. influenzae DNA fragments A, H, I, and B, all of which are contiguous on the physical map. The "late" region of the viral genome includes the plus strands of fragments, C, D, E, F, G, J, K and portions of A and B. Transcription of stable species of "early" lytic RNA is initiated in fragment A and proceeds toward fragment B; transcription of "late" RNA occurs in the opposite direction on the plus strand of viral DNA. (Martin, Khoury, Nathans [Johns Hopkins Univ.]).

Defective Forms of SV40 Provide Defined Partial Copies of the SV40 Genome

Characterization of the type of defective SV40 which forms a "light" band in CsCl has indicated that specific deletions occur in the supercoiled form of viral DNA. One defective preparation consists of encapsidated viral DNA which is 80% the size of wild-type DNA and contains 55% of the genetic information of standard SV40. This defective form of SV40 can replicate its DNA in monkey cells although T and V antigens and progeny virions fail to appear. Stable species of symmetrically transcribed RNA are present. (Martin, Fareed).

Regulation of SV40 Transcription Has Been Studied In Vitro

It has previously been shown that chromatin is a restricted template; only a small portion of the chromatin DNA is available for transcription. Studies on the template activity of partially dehistonized chromatins and reassociated DNA-histone complexes have indicated that histone is the major repressor substance. When purified chromatin isolated from a SV40 transformed mouse cell line (11A8) was examined, the template activity is about 17% that of purified DNA confirming that more than 80% of chromatin DNA is repressed presumably by its association with chromosomal

proteins. SV40 DNA was employed as a molecular probe to study the viral specific RNA transcribed by E. coli RNA polymerase from chromatin of SV40-transformed cells. The presence of SV40 specific RNA sequences was detected by annealing the RNA with separated strands of $^{32}$P-SV40 DNA fragments in solution and the hybrid formed was analyzed by hydroxyapatite chromatography. The RNA transcript of the integrated SV40 genome appears to be assymmetric; it hybridized to a much greater extent to the minus than to the plus strand of SV40 DNA. This is similar to the pattern observed in vivo. The viral specific RNA also shares sequences with "early" lytic SV40 specific RNA, and is, in general, similar to that present in SV40-transformed cells. (Shih, Khoury, Martin)

A Procedure Has Been Devised to Isolate Transformed Cell DNA

SV40 cRNA, assymmetrically transcribed from SV40 DNA I by E. coli RNA polymerase, was chemically linked to a cellulose matrix. The SV40 RNA-cellulose was then packed in the reassociation chamber portion of a continuous hybridization system. The cellulose matrix was maintained at 37°C to allow efficient DNA-RNA interaction in the stationary phase, while unreacted DNA and any reassociated DNA in the mobile phase was denatured at 90°C and then recirculated into the hybridization chamber. Between 40 and 45% of fragmented $^{32}$P-SV40 DNA (very close to the theoretical 50%) was removed from the circulating solution. In the presence of $10^4$-fold excess of sheared E. coli DNA, the same amount of $^{32}$P-SV40 DNA was isolated with negligible contamination by the unrelated bacterial DNA. The efficiency and selectivity of this method will permit the isolation of integrated SV40 DNA sequences from virus-transformed cells. (Shih, Martin)

Cleavage of SV40 Virus DNA by the Hemophilus Parainfluenza Restriction Enzyme

Purification of the enzyme was facilitated by development of a rapid assay which measures the conversion of closed circular, duplex DNA to a cleaved form which can be detected by differential adsorption to hydroxyapatite after denaturation. SV40 DNA is cleaved by this restriction enzyme into 3 major fragments representing 43, 35 and 20% of the original DNA. These pieces are easily separated on 1.5% agarose gels from which they are eluted and further digested with exonuclease III or the exonuclease associated with T4 DNA polymerase. The exonuclease treated pieces can be sized on acrylamide-agarose gels so that different length single stranded DNA pieces with the original 5' end of the restriction enzyme fragments can be obtained. These pieces, of any desired length, can be used in liquid hybridization experiments (DeFilippes)

Capsid Proteins of SV40 Plaque Mutants Reveal Electrophoretic Differences

It is possible to distinguish large and small plaque mutants of SV40 by the isoelectric focusing technique and capsid proteins of the two mutants can also be differentiated in the same way. An explanation for the difference in electrophoretic charge in the capsid proteins of small and large plaque SV40 mutants was obtained by peptide analyses. A single

peptide difference was found in the peptide maps obtained from the tryptic digests of the capsid proteins of the SV40 mutants after separation by electrophoresis on thin layer and detection by autoradiography. Examination of the number of peptides obtained by fingerprinting of SV40 capsid protein reveals only half the number of spots predicted from amino acid analyses of the lysine and arginine residues. These results suggest that the SV40 capsid protein is composed of two identical subunits. (Barban)

Phosphorylated Histones are Altered as Cells Enter Mitosis

Cultured Chinese hamster and other cell types contain 2 major histone phosphokinase activities able to phosphorylate F1 (Lysine-rich) histone. These two activities designated KI and KII, were readily separated by G-200 gel filtration. KI differs from KII by several criteria. KII is 90,000 M.W., Cyclic AMP independent, rapidly turned over in vivo, low Km for ATP, and phosphorylates F1-histone at several unique sites. Comparative examination of metaphase arrested (M) and counterpart interphase (I) cells for these two activities reveals that the KII enzyme is responsible for the overall high activity in M-arrested cells. Pulse labeling of cells with $^{32}P$ during traverse of the G2-M phase of the cell cycle reveals an in vivo tryptic phosphopeptide pattern unique to M cell F1 histone. Seven major phosphopeptides derived by in vitro phosphorylation of F1 with the KII enzyme correspond to these M cell specific phosphorylation sites observed in vivo. It can be concluded that KII activity predominates during the G2 transition and that F1 is its natural in vivo substrate. The phosphorylated form of F1 that exists at metaphase due to the action of the KII phosphokinase is preserved until cells are allowed to enter G1. (Lake, Salzman)

Chromatin Structure is Preserved during Mitosis

If the functioning genes of a given cell are regulated by the proteins associated with chromosomal DNA, the restriction imposed on chromatin DNA should be transmitted quite faithfully to daughter cells. This question was examined by studying changes of DNA-histone interaction and nucleohistone structure in the process of mitosis as manifested by condensation of chromosomes at the metaphase. Chromatins were isolated from metaphase Chinese hamster cells arrested by vinblastine as well as the remaining interphase cells. The structure of nucleohistone was studied by thermal denaturation profiles and circular dichroism spectroscopy. The results indicate that the elementary structure of nucleohistone, such as the histone stabilization of DNA double helix and supercoiled configuration of the nucleohistone fiber, is preserved during mitosis, thereby ensuring that the same chromatin structure is passed to daughter cells. (Shih, Lake)

The Role of Helper Viruses in Adenovirus-Associated Virus Replication
The AAV Genome is Incompletely Transcribed

Adenovirus-associated viruses (AAV) are small, DNA-containing viruses that multiply only in cells which are co-infected with an adenovirus. Herpesviruses also provide some function(s) required for AAV multiplication;

AAV antigen(s) is synthesized but an increase in AAV infectivity or particles has not been observed. Nucleic acid hybridization procedures were used to measure the extent of transcription of adenovirus-associated virus (AAV) deoxyribonucleic acid (DNA) in KB cells in the presence of either adenovirus or herpes simplex virus as the helper. These experiments demonstrated that in the presence of either type of helper virus, only one strand of AAV DNA (the thymidine-rich or "minus" strand) is transcribed in vivo. However, it was found that only 70-80% of this strand appears to be transcribed in vivo. Furthermore, studies with minus strands employing hydroxyapatite chromatography and nuclease $S_1$, which specifically degrades single-stranded DNA, indicated that up to 20% of the minus strand may be self-complementary. It seems likely that these self-complementary sequences account for the bulk of that portion of the minus strand (20-30%) which is not transcribed in vivo.

The Terminal Structure of AAV DNA Resembles that of its Helper Virus

At least 70% of plus or minus strands of adenovirus-associated virus DNA contain self-complementary sequences at or near their termini. Self-annealing of these sequences generates circular molecules that are closed by duplex, hydrogen-bonded segments. The self-annealed segments are sensitive to exonuclease III and have a thermal stability comparable to that of double-stranded DNA molecules. Length measurements of double-stranded adenovirus-associated virus DNA molecules show a bimodal distribution, with the larger component being 10% shorter than SV40 DNA.

The presence of self-complementary terminal sequences in single-stranded molecules of viral DNA has been observed previously only with DNA from adenoviruses. It is thus especially notable that adenovirus-associated virus replication is unconditionally dependent on a helper adenovirus. The terminal self-complementary sequences may well play a role in viral DNA replication. (Koczot, Carter, Garon, Rose)

Adenovirus Provides Separate Helper Functions for AAV DNA and RNA Synthesis

The temporal relationships of viral DNA and RNA synthesis in KB cells co-infected with adenovirus-associated virus type 2 (AAV-2) and adenovirus type 2 (Ad2) as the helper have been analyzed. In cells simultaneously infected with AAV-2 and Ad2 both AAV and Ad DNA synthesis began at 6.5 to 7.5 hours. AAV RNA synthesis began at 9-10 hours whereas Ad RNA synthesis could be detected at 2-3 hours and increased greatly in rate at 10 hours. When cells were infected with Ad2 for 10 hours prior to infection with AAV-2, the interval between AAV DNA and RNA was abolished and synthesis of both began within 3 to 4 hours after AAV infection. Furthermore, in a simultaneous infection AAV transcription did not occur when viral DNA synthesis was blocked with fluorodeoxyuridine (FUDR). These findings suggest that (i) AAV DNA and RNA synthesis require separate helper functions which appear sequentially after Ad infection, (ii) both AAV and Ad DNA synthesis may be initiated by the same factor(s), (iii) the initiation of AAV RNA synthesis may depend upon a factor which controls late adenovirus transcription, and (iv) initiation of AAV

RNA synthesis requires viral DNA synthesis. (Rose, Carter)

Heteroduplex Analysis Provides A Molecular Basis for Serologic Interrelationship of Adenoviruses

When DNA from adenovirus serotypes was mixed, denatured and renatured characteristic heteroduplex molecules were formed. These molecules, composed of one strand from one serotype and one strand from the other serotype, contained one or more detectable regions of strand separations (or heterology) depending upon denaturing conditions and the combination of type specific DNA preparations. The heteroduplex patterns in heteroduplexes constructed with DNA from nononcogenic serotypes (Ad 1, 2 and 5) were relatively simple and nearly similar, whereas both simple and complex patterns were seen in heteroduplexes prepared with DNA from weakly oncogenic serotypes (Ad 3, 7 and 21). Only complex heterology patterns were found in heteroduplexes prepared with DNA from highly oncogenic serotypes (Ad 12, 18 and 31). As expected, the most extensive heterologies were observed in heteroduplexes prepared with DNA from serotypes belonging to different serologic groups. Within groups, heteroduplex molecules contained two specific regions in which heterologies were most marked. These regions appeared to map at similar locations among heteroduplexes from all groups. Furthermore, it was found that the integrated SV40 virus DNA in an Ad7-SV40 hybrid genome was situated in one of these regions. (Garon, Rose)

Solubilization of the Poxviral Core and the Isolation of 5 Viral Enzymes Has Been Achieved

Polynucleotide-dependent nucleotide phosphohydrolases. Two polynucleotide-dependent nucleotide phosphohydrolases (NPH I and NPH II) were discovered in vaccinia virus cores. Both enzymes were purified several hundred fold and had molecular weights of 68,000 daltons. NPH I hydrolyzed only ATP to an appreciable extent whereas NPH II hydrolyzed all 4 ribonucleoside triphosphates. The polynucleotide requirement of NPH I was met with denatured DNA, synthetic polydeoxyribonucleotide hybrids. Polydeoxyribonucleotide-polyribonucleotide hybrids but not with completely native DNA, single stranded homopolymers or polyribonucleotide hybrids. In contrast all of the above polynucleotides except for completely native DNA and RNA effectively stimulated NPH II activity. Enzymes from animal cells or viruses similar to NPH I or NPH II have not previously been described and their biological roles remain to be determined. (Paoletti, Moss)

Protein kinase. A protein kinase activity was discovered in vaccinia virions by measuring the incorporation of $^{32}P$ from $\gamma$-$^{32}P$-ATP onto serine or threonine residues of proteins. The protein kinase activity was shown to consist of heat stable and heat labile protein components which were separated and partially purified by column chromatography. These results suggest that a unique type of regulatory process may be involved in the activation of the viral protein kinase. (Kleiman, Moss)

Deoxyribonuclease. A deoxyribonuclease, present in vaccinia virions,

was purified and characterized. The deoxyribonuclease was purified several hundred fold and shown to have a subunit molecular weight of 50,000 daltons. The purified enzyme has both endonuclease and exonuclease activities but is entirely specific for single-stranded DNA. Short single-stranded regions within a duplex structure are cleaved and thus SV40 DNA form I was converted to form II. The enzyme may play a role in the synthesis or processing of vaccinia DNA. (Rosemond, Moss)

Polyadenylate polymerase. A poly A polymerase was extracted from vaccinia virus cores and assayed by measuring the formation of polyadenylic acid from radioactively labeled ATP. Purification was achieved by column chromatography and the enzyme was free of detectable RNA polymerase activity. Stimulation by poly dA:poly dT and inhibition by ethidium bromide and proflavine appear to be unique properties of this viral enzyme. (Moss, Rosenblum, Paoletti)

Several Types of Post-Translational Modifications of Poxviral Proteins Have Been Identified

Formation of structural proteins from higher molecular weight precursors. High molecular weight precursors of the 2 major vaccinia viral core proteins were identified by peptide analysis. The precursors were labeled with $^{35}S$-methionine by blocking their cleavage with rifampicin, a drug previously shown to specifically interrupt vaccinia virus assembly. (Rosenblum, Moss)

Glycosylation of virion proteins. The glycoprotein was purified according to molecular weight by SDS-polyacrylamide gel electrophoresis and then separated from 3 similar molecular weight proteins by SDS-hydroxylapatite chromatography. Radioisotopic labeling experiments suggested that both the polypeptide and carbohydrate portions were formed primarily after the onset of viral DNA synthesis. (Rosenblum, Moss)

Phosphorylation of virion proteins. The recent discovery of a protein kinase activity in vaccinia virions led to a search for a viral protein which was phosphorylated in-vivo. Vaccinia virus was radioactively labeled by infecting cells in the presence of $^{32}Pi$. A phosphoprotein was isolated and appeared to be a specific viral component induced after infection. Tryptic peptide analysis of the purified protein suggested that a single serine residue was phosphorylated. The high selectivity of phosphorylation suggested that the process has a specific function. (Rosemond, Moss)

An Intermediate Involved in Kilham Rat Virus (KRV) DNA Synthesis Has Been Characterized

In order to study the DNA replicative intermediates involved in KRV synthesis, the viral DNA was made radioactive by incorporation of $^{3}H$-thymidine. Kilham rat virus (KRV) contains within the virion a linear single-stranded DNA molecule of molecular weight about $1.6 \times 10^{6}$. Within

60 min after infection of cells 28 to 42% of the parental viral DNA is converted to a new form. This new DNA form is believed to be double stranded and linear by a number of criteria.

The growth characteristics of KRV and its effect on cellular macromolecular synthesis were also determined. Virus infection caused an immediate inhibition of cellular DNA synthesis in the infected cell lasting about 6 hours. Detectable viral DNA synthesis started at 6-8 hours after infection. DNA synthesis in the infected cell started to increase at that time and by 16 hours after infection was twice that found in the uninfected control cell. The amount of protein and RNA synthesized in the infected cells were similar. (L. Salzman)

RNA Tumor Virus Genetic Information is Contained in Animal Cells

Approximate 11-13 copies of murine leukemia virus (MLV) specific DNA are present in normal mouse cells; less than 0.4 copy/cell was detected in chicken, monkey or human cells. Infection of mouse cells with MLV does not result in the addition of detectable amounts of MLV DNA. Infection of human cells, however, with MLV leads to the incorporation of 3 additional copies of the leukemia virus genome. The acquired viral genetic information is apparently covalently linked to the high molecular weight fraction of human DNA. (Martin, Milstien)

Serial No. NIAID -80A
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biochemical Characterization of Nucleic Acids of Transformed and Virus Infected Cells

Previous Serial No.: Same

Principal Investigators: Dr. Malcolm Martin
Dr. George Khoury
Dr. Julie Milstien
Ms. Janet Byrne

Cooperating Units: Dr. Kenneth Takemoto, LBV, NIAID
Dr. Stuart Aaronson VCB, NCI
Dr. Daniel Nathan, Department of Microbiology, Johns Hopkins School of Medicine, Baltimore, Md.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 58/12 | | | 58/12 |
| Professional: | 30/12 | | | 30/12 |
| Other: | 28/12 | | | 28/12 |

Project Description:

Objectives:

To elucidate the process of viral DNA transcription under conditions of productive and transforming infection.

To examine the mechanisms involved in the integration of viral genetic material in the animal cell genome.

To detect and characterize RNA tumor virus genetic material in normal and transformed cells by utilizing labeled in vitro DNA made with the virus-associated RNA dependent DNA polymerase.

Methods Employed:

Simian virus (SV) 40 was propagated in green monkey cells and purified by isopyonic centrifugation in CsCl. SV40 DNA was isolated from purified virions and viral specific RNA was obtained from infected cells. Viral DNA was analyzed by isopyonic centrifugation, velocity rate sedimentation in neutral and alkaline conditions, sensitivity to specific restriction endonucleases and reassociation kinetics. SV40 specific RNA, purified from lytically infected and transformed cells, was characterized by its ability to react with the separated strands of viral DNA.

Major Findings:

SV40 DNA Transcription: We had previously shown that during lytic infection of green monkey cells, 30-35% of the minus DNA strand 65-70% of the plus DNA strand are expressed. Similar studies, carried out with 12 SV40 transformed cell lines, have indicated that transcription occurs predominantly from the minus strand of the integrated viral genome. In 10 of 12 lines examined 30-50% of the minus viral DNA strand is expressed; in 2 lines, 65-75% is transcribed. The portion of the minus DNA strand expressed in all of the transformed cell lines tested also contains the viral genetic information transcribed during the early phase of the lytic cycle. Transcription of the plus DNA strand was detected in only 3 of 12 lines; less than 10% was expressed in all cases.

In collaboration with Dr. Daniel Nathans we have been able to topographically locate the "early" and "late" regions of SV40 DNA on the physical map of the viral genome. The "early" segment is included in DNA sequences corresponding to the minus strands of H. influenzae DNA fragments A, H, I, and B, all of which are contiguous on the physical map. The "late" region of the viral genome includes the plus strands of fragments, C, D, E, F, G, J, K and portions of A and B. Transcription of stable species of "early" lytic RNA is initiated in fragment A and proceeds toward fragment B; transcription of "late" RNA occurs in the opposite direction on the plus strand of viral DNA.

Defective Forms of SV40: Characterization of the type of defective SV40 which form a "light" band in CsCl has indicated that specific deletions occur in the supercoiled form of viral DNA. One defective preparation consists of encapsidated viral DNA which is 80% the size of wild-type DNA and contains 55% of the genetic information of standard SV40. This defective particle can replicate its DNA in monkey cells although T and V antigens and progeny virions fail to appear. Stable species of symmetrically transcribed RNA are present.

RNA Tumor Virus Genetic Information in Animal Cells. Approximate 11-13 copies of murine leukemia virus (MLV) specific DNA are present in normal mouse cells; less than 0.4 copy/cell was detected in chicken, monkey or human cells. Infection of mouse cells with MLV does not result in the addition of detectable amount of MLV DNA. Infection of human cells, however, with MLV leads to the incorporation of 3 additional copies of the leukemia

virus genome. The acquired viral genetic information is apparently covalently linked to the high molecular weight fraction of human DNA.

Significance to Biomedical Research and the Program of the Institute:

The ultimate goal of this project is to understand the integration of viral genetic material into the mammalian chromosome and the relationship of this process to oncogenesis. The determination of the time course of integration, the number of copies of viral DNA present in cells undergoing transforming infection and the nature of the integration site in the animal cell genome may permit one to separate the various steps involved and possibly interrupt these molecular events with appropriate chemotherapeutic agents.

We are also interested in delineating those viral genes necessary to induce and/or maintain the transformed state. Utilizing the separated strands of SV40 DNA, we will be able to characterize those viral gene products that are present in tumor cells from those required for SV40 productive infection. A more thorough understanding of the control mechanisms which modulate viral DNA transcription may enable one to regulate the type of cellular response to these agents.

Proposed Course of the Project:

Emphasis will be placed on understanding the mechanisms of integration of both RNA and DNA tumor virus genetic material into the host chromosome(s). Mammalian DNA from cells undergoing both productive and transforming infection will be examined to determine when stable integration occurs. We are also in the process of characterizing the viral-specific genetic information shared by RNA tumor viruses and normal cells. Utilizing the separated strands of SV40 DNA we are examining the pattern of viral transcription in a series of transformed cell lines derived from several different mammalian species.

Honors and Awards:

Invited lectures at:

NSF symposium entitled "Transcription and Translation," Palo Alto, California. (6/72)

Roche Institute of Molecular Biology, Nutley, New Jersey.

University of Minnesota symposium entitled, "Eucaryotic DNA," (9/72).

IV hepetit colloquiem, "Possible Episomes in Eukaryotes," Cocayoc, Mexico. (11/72)

University of Illinois Medical School, Chicago, Illinois. (2/73)

UCLA Symposium in Molecular Biology, Squaw Valley, California, 3/73

Institute for Medical Research, Camden, New Jersey. (3/73)

Publications:

Khoury, G. and Martin, M. A.: Comparison of SV40 DNA transcription in vivo and in vitro. Nature New Biology 238: 4-6, 1972.

Khoury, G., Byrne, J. C. and Martin, M. A.: Patterns of Simian Virus 40 DNA transription after acute infection of permissive and nonpermissive cells. Proc. Nat. Acad. Sci. USA 69: 1925-1928, 1972.

Khoury, G., Byrne, J. C., Takemoto, K. K. and Martin, M. A.: Patterns of Simian Virus 40 DNA Transcription: II. In transformed cells. J. Virol. 11: 54-60, 1973.

Gelb, L. D. and Martin, M. A.: Simian Virus 40 DNA integration within the genome of virus-transformed mammalian cells. Virology 51: 351-357 (1973).

Serial No. NIAID-80G
1. Biology of Viruses
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Characterization and Biosynthesis of Viral Enzymes and Structural Proteins.

Previous Serial Number: Same

Principal Investigator: Dr. Bernard Moss

Other Investigators: Dr. Enzo Paoletti
Dr. Hortencia Rosemond
Dr. Jay Kleiman
Ms. Edith Rosenblum
Mr. Norman Cooper

Cooperating Units: None

Man Years:

| | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 90/12 | | | 90/12 |
| Professional: | 60/12 | | | 60/12 |
| Other: | 30/12 | | | 30/12 |

Project Description

Objectives:

1. Identification, isolation and characterization of viral enzymes and structural proteins with the aim of elucidating their roles in viral replication.

2. Determination of regulatory mechanisms involved in the biosynthesis of viral proteins.

3. Analysis of post-translational modifications of viral proteins and their relationship to enzyme function and virion assembly.

4. Demonstration of the effects of antiviral drugs on the function and biosynthesis of viral proteins and on the assembly of virions.

Methods Employed:

Vaccinia virus was grown in HeLa cells and purified by density gradient sedimentation. Biosynthesis of macromolecules was determined by incorporation of radioactive precursors and separation by gel electrophoresis, gel filtration, column chromatography or rate zonal and equilibrium centrifugation. Enzymes were purified by column chromatography and were assayed by measuring the transformation of radioactively labeled precursor molecules. Structural analysis of purified macromolecules was made by enzymatic and chemical hydrolysis followed by electrophoretic and chromatographic analysis.

Major Findings:

A. Five enzymes were isolated from purified vaccinia virions.

1. Polynucleotide-dependent nucleotide phosphohydrolases. Two polynucleotide-dependent nucleotide phosphohydrolases (NPH I and NPH II) were discovered in vaccinia virus cores. The enzymes were solubilized with sodium deoxycholate and assayed by measuring the formation of ribonucleoside diphosphates from radioactively-labeled triphosphates. Both enzymes were purified several hundred fold and had molecular weights of 68,000 daltons. Both NPH I and NPH II required polynucleotides, divalent cations and neutral pH values for maximal enzymatic activities. NPH I hydrolyzed only ATP to an appreciable extent whereas NPH II hydrolyzed all 4 ribonucleoside triphosphates. The polynucleotide requirement of NPH I was met with denatured DNA, synthetic polydeoxyribonucleotide hybrids, polydeoxyribonucleotide-polyribonucleotide hybrids but not with completely native DNA, single stranded homopolymers or polyribonucleotide hybrids. In contrast all of the above polynucleotides except for completely native DNA and RNA effectively stimulated NPH II activity. Enzymes from animal cells or viruses similar to NPH I or NPH II have not previously been described and their biological roles remain to be determined.

2. Protein kinase. A protein kinase activity discovered in vaccinia virions last year, has now been solubilized and characterized. Enzyme activity was assayed by measuring the incorporation of $^{32}P$ from $\gamma$-$^{32}P$-ATP onto serine or threonine residues of proteins. Maximal activity was dependent on divalent cations, a reducing agent and added basic proteins. The protein kinase activity of solubilized virions was further shown to consist of heat stable and heat labile protein components which were separated and partially purified by column chromatography. These results suggest that a unique type of regulatory process may be involved in the activation of the viral protein kinase.

3. Deoxyribonuclease. A deoxyribonuclease, previously shown to be present in vaccinia virions, was purified and characterized. The enzyme was extracted from vaccinia virus cores with sodium deoxycholate and assayed by measuring the hydrolysis of radioactively

labeled cellular or viral DNA. The deoxyribonuclease was purified several hundred fold and shown to have a subunit molecular weight of 50,000 daltons. The purified enzyme has both endonuclease and exonuclease activities but is entirely specific for single-stranded DNA. Short single-stranded regions within a duplex structure are cleaved and thus SV40 DNA form I was converted to form II. The enzyme may play a role in the synthesis or processing of vaccinia DNA.

4. Polyadenylate polymerase. A poly A polymerase, recently found in vaccinia virions, was isolated and characterized. The enzyme was extracted from vaccinia virus cores with sodium deoxycholate and assayed by measuring the formation of polyadenylic acid from radioactively labeled ATP. Purification was achieved by column chromatography and the enzyme was free of detectable RNA polymerase activity. Stimulation by poly dA:poly dT and inhibition by ethidium bromide and proflavine appear to be unique properties of this viral enzyme.

B. Post-translational modifications.

1. Formation of structural proteins from higher molecular weight precursors. Precursors of the 2 major vaccinia viral core proteins were identified by peptide analysis. The precursors were labeled with $^{35}$S-methionine by blocking their cleavage with rifampicin, a drug previously shown to specifically interrupt vaccinia virus assembly. Both precursors and structural proteins were purified by SDS-polyacrylamide gel electrophoresis, digested with trypsin and analyzed by thin layer electrophoresis and chromatography. A core protein with a molecular weight of 68,000 was formed from a 105,000 dalton precursor and a core protein of 62,000 daltons was formed from a 74,000 dalton precursor.

2. Glycosylation of virion proteins. A 2 step procedure was devised for the purification of the vaccinia virion glycoprotein. The glycoprotein was purified according to molecular weight by SDS-polyacrylamide gel electrophoresis and then separated from 3 similar molecular weight proteins by SDS-hydroxylapatite chromatography. Peptide maps of tryptic digests indicated that the proteins separated by chromatography had different amino acid sequences. The molecular weight of the virion glycoprotein, determined by electrophoresis at several gel concentrations was approximately 38,500. Radioisotopic labeling experiments were carried out to determine the relative time of synthesis of the glycoprotein. These studies suggested that both the polypeptide and carbohydrate portions were formed primarily after the onset of viral DNA synthesis.

3. Phosphorylation of virion proteins. The recent discovery of a protein kinase activity in vaccinia virions led us to search for a viral protein which was phosphorylated in-vivo. Vaccinia virus

was radioactively labeled by infecting cells in the presence of $^{32}Pi$. A phosphoprotein was isolated from purified de-lipidated virions by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The phosphoprotein appeared to be a specific viral component induced after infection. More than 60 per cent of the phosphoprotein was associated with viral cores. The electrophoretic mobility of the protein suggested that it has a molecular weight of 11,000 to 12,000 daltons. Phosphoserine was liberated by acid hydrolysis and identified by electrophoresis with known standards. Tryptic digests of the purified phosphoprotein were analyzed by 2 dimensional electrophoresis and chromatography on thin layer cellulose plates and a single major phosphopeptide was resolved. The high selectivity of phosphorylation suggested that the process has a specific function.

Significance to Biomedical Research and the Program of the Institute:

Enzymes have been found associated with both RNA and DNA-containing virions but very few have been isolated and characterized. The solubilization of the vaccinia virus core and the purification of 5 separate enzymes represents a significant advance in this field. We should now be able to fully characterize the enzymes and possibly learn their precise role in virus replication. On the basis of preliminary studies, it appears that these viral enzymes have unique properties which distinguish them from previously described cellular enzymes and thus are potential targets of anti-viral compounds.

The formation of viral proteins by cleavage of higher molecular weight precursors, first thought to be unique to certain RNA viruses, has now been conclusively shown to occur during the replication of vaccinia, a DNA virus and thus may be of widespread occurrence. It is of further interest that rifampicin, a specific anti-viral drug, interrupts virion assembly and blocks the cleavage process.

Proposed Course of the Project:

During the next year we expect to further purify and characterize the 5 isolated virions enzymes and to study their biosynthesis and assembly into virions. It is anticipated that the drug rifampicin will be a unique tool for the latter studies.

Honors and Awards:

Editorial Board of Journal of Virology

Editorial Board of Antimicrobial Agents and Chemotherapy

Invited participant at Gordon Research Conference on Animal Cells and Viruses.

Publications:

Moss, B. and Goldblum, N.: Antiviral effects of rifampicin and related compounds. In J. L. Melnick (Ed.): International Virology 2, 291-299, Basel S. Karger, 1972.

Moss, B., Rosenblum, E. N., Grimley, P. M. and Mims, S. J.: Rifamycins: Modulation of specific anti-poxviral activity by small substitutions on the piperazinyliminomethyl side chain. Antimicrob. Ag. and Chem. 2: 181-185, 1972.

Paoletti, E. and Moss, B.: Protein kinase and specific phosphate acceptor proteins associated with vaccinia virus cores. J. Virol. 10: 417-424, 1972.

Paoletti, E. and Moss, B.: Deoxyribonucleic acid-dependent nucleotide phosphohydrolase activity in purified vaccinia virus. J. Virol. 10: 866-868, 1972.

Moss, B. and Rosenblum, E. N.: Hydroxylapatite chromatography of protein-sodium dodecyl sulfate complexes. A new method for the separation of polypeptide subunits. J. Biol. Chem. 247: 5194-5198, 1972.

Rosemond, H. and Moss, B.: Phosphoprotein component of vaccinia virions. J. Virol. In press.

Moss, B.: Rifamycin SV derivatives. In W. Carter (Ed.): Selective inhibitors of viral functions, Cleveland, CRC Press, in press.

Serial No. NIAID-80H
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Replication of the Parvovirus, KRV

Principal Investigator: Dr. Lois A. Salzman

Other Investigators: Mr. Wesley White
Ms. Louese McKerlie

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 28/12 | | | 28/12 |
| Professional: | 12/12 | | | 12/12 |
| Other: | 16/12 | | | 16/12 |

Project Description:

Objectives:

1. To determine the fate of Kilham rat virus DNA after infection of a susceptible cell and the mechanism of the virion DNA replication.

2. To examine the synthesis of viral RNA after infection of a susceptible host.

3. To solubilize, purify and characterize any enzymes associated with the purified virions.

Methods Employed:

In order to study the DNA replicative intermediates involved in KRV synthesis, the viral DNA was made radioactive by incorporation of $^{3}$H-thymidine. A new form of viral DNA was found to be synthesized after infection and is believed to be double stranded

and linear on the basis of its sedimentation in neutral and alkaline sucrose gradients elution from hydroxyapatite columns, its buoyant density in CsCl gradients and appearance in the electron microscope.

Comparison of DNA, RNA and protein synthesis in infected and uninfected cells was determined by the incorporation of the appropriate radioisotope precursor into acid insoluble material. Viral DNA synthesis was also studied with the aid of the inhibitor of DNA synthesis, 5-fluorodeoxyuridine (FIDR). The growth cycle of the virus in a permissive cell was followed by formation of plaques on a rat nephroma monolayer.

Studies on viral RNA synthesis were accomplished by incorporation of the radioisotope $^{3}$H-uridine into the RNA. The RNA was extracted from the infected cell by a procedure involving hot phenol and then hybridized to DNA bound to a membrane filter.

DNA polymerase was solubilized from purified virions with the aid of deoxycholate (DOC) and the non-ionic detergent, NP-40. The enzyme was purified by diethylaminoethyl cellulose hydroxylapatite phosphocellulose and DNA cellulose column chromatography. The enzyme assay involves the incorporation of $^{3}$H-deoxyadenosine triphosphate into acid insoluble material.

Major Findings:

An intermediate involved in viral DNA synthesis has been characterized. Kilham rat virus (KRV) contains within the virion a linear single-stranded DNA molecule of molecular weight about $1.6 \times 10^{6}$. Within 60 min after infection of cells 28 to 42% of the parental viral DNA is converted to a new form. This new DNA form is believed to be double-stranded and linear by a number of criterial. The intermediates in DNA replication by other mammalian parvoviruses have not been characterized. The bacteriophage members of the parvovirus group contain single-stranded, circular DNA in the virion and have a double-stranded circular replicative intermediate.

Effect of KRV on cellular DNA, RNA and protein synthesis. Some of the growth characteristics of KRV and its effect on cellular macromolecular synthesis were determined. Virus infection caused an immediate inhibition of DNA synthesis in the infected cell lasting about 6 hours. Detectable viral DNA synthesis started at 6-8 hours after infection. DNA synthesis in the infected cell started to increase at that time and by 16 hours after infection was twice that found in the uninfected control cell. The amount of protein and RNA synthesized in the infected cells were similar.

Viral RNA synthesized from parental strand of virion DNA. Preliminary results from studies on viral RNA synthesis after infection of a permissive host indicate that the viral RNA specifically hybridizes with the input single-stranded virion DNA. Bacteriophages from the parvovirus group have been shown to use the complementary strand from the RF form of the DNA for RNA synthesis. Future studies will reveal the amount of the viral genome used for transcription, the size of the viral RNAs and hopefully the proteins synthesized by these RNAs.

Viral RNA synthesized from parental strand of virion DNA. Preliminary results from studies on viral RNA synthesis after infection of a permissive host indicate that the viral RNA specifically hybridizes with the input single-stranded virion DNA. Bacteriophages from the parvovirus group have been shown to use the complementary strand from the RF form of the DNA for RNA synthesis. Future studies will reveal the amount of the viral genome used for transcription the size of the viral RNAs and hopefully the proteins synthesized by these RNAs.

KRV associated DNA polymerase solubilized and partially characterized. The DNA polymerase found consistantly associated with the purified virus has been solubilized. A procedure for the purification of the enzyme has been worked out and some of the enzyme characteristics have been determined. The enzyme is very unstable when purified and methods tried to stabilize it have so far been limited in value. Preparation of larger amounts of the enzyme and further alteration in conditions are expected to be successful in more complete enzyme characterization.

Significance to Bio-medical Research and the Program of the Institute. Kilham rat virus (KRV) first isolated from a rat sarcoma is a member of the parvovirus group which is ubiquitous and widespread. Parvoviruses are found in many mammals, birds as well as lepidoptera and bacteria. H1 virus, closely related to KRV, and the defective virus AAV are human parvoviruses. Parvoviruses contain a very small amount of DNA capable of coding for a very few proteins. With this small amount of information, they replicate and cause cell lysis. Little is known about the method of virus control of the mammalian cell and virion DNA replication. Using KRV as a model it may be possible to determine some of the important aspects of the virus-cell interaction.

The parvoviruses are also of interest because of their interaction with oncogenic viruses. KRV has been reported to suppress induction of leukemia in rats by the RNA maloney virus. (Bergs, Cancer Res. 29: 1669, 1969). H1 can reduce the incidence of spontaneous and induced tumors in hamsters. The adeno-associated virus (AAV) inhibits transformation of hamster embryo cells and induction of tumors *in vivo* by the oncogenic adenovirus 12. An understanding of the replicative cycle of the parvovirus may aid in the understanding of this suppression of some cancer manifestations.

KRV and H1 are capable of causing latent or inapparent infections. The presence of latent viruses in cells, their effect on the cell and the cause of activation of these latent viruses are of considerable interest to those studying malignancy and other disease states. The parvoviruses because of their apparent simplicity may serve as excellent models for the study of latent diseases.

Proposed Course of the Project:

1. Further purification and characterization of the viral associated enzymes will be undertaken. I hope to more closely define the role of the DNA polymerase associated with the virion and to determine its role in virus replication.

Proposed Course of the Project:

1. Further purification and characterization of the viral associated enzymes will be undertaken. I hope to more closely define the role of the DNA polymerase associated with the virion and to determine its role in virus replication.

2. Investigation of the viral RNA synthesized by the infected cell will be investigated further. The size and number of the viral RNAs necessary for virus replication and cell destruction will be determined.

3. To study further the synthesis of viral DNA in an infected cell. To determine if cellular DNA is completely blocked after infection and to gain some insight into how this control of the cell is exerted.

4. To study KRV as a latent viral infection. To determine if the virus can be integrated into the host cell chromosome and to investigate the control mechanisms involved in viral latency and infection.

Honors and Awards:

Invited lecture - Georgetown University School of Medicine - December 1972.

Publications:

1. Salzman, L. A., White, W. L., McKerlie, L.: Growth characteristics of Kilham Rat Virus and its Effect on Cellular Macromolecular Synthesis. J. Virology 10: 573-577, 1972.

2. Salzman, L. A., White, W.: In Vivo Conversion of the Single-Stranded DNA of the Kilham Rat Virus to a Double-Stranded Form. J. Virology 11: 299-305. 1973.

Serial No. NIAID-84B
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies with Isolated Metaphase Chromosomes

Previous Serial Number: Same

Principal Investigator: R. S. Lake
N. P. Salzman

Other Investigator: T. Y. Shih

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 18/12 | | | 18/12 |
| Professional: | 12/12 | | | 12/12 |
| Other: | 6/12 | | | 6/12 |

Project Description:

Objectives:

(1) To separate and characterize a histone phosphokinase found to be elevated above basal activity in metaphase of cultured animal cells.

(2) Examine the in vitro and in vivo product of this histone phosphokinase in comparison with other kinases of the same cell type.

(3) Define temporal relationships between the occurrence of this phosphorylation - dephosphorylation during traverse of the G2→M→G1 cell cycle stages.

Methods Employed:

Vinblastine or Colcemid-arrested metaphase cells were obtained from Hela S-3, Vero (African Green Monkey) and Chinese Hamster (V-79) cultures. Lysine-rich histone from these cells was examined for its degree of phosphorylation by polyacrylamide gel electrophoresis on urea-acetic acid gels. Protein phosphokinases were extracted from these metaphase cells and their counterpart interphase cells and separated by ammonium sulfate precipitation, G-200 Sephadex chromatography, and ion exchange chromatography. Specificity of in vitro and in vivo phosphorylation reactions was determined by radioautography of one-dimensional tryptic ($^{32}P$) phosphopeptide electropherograms.

Major Findings:

Cultured Chinese hamster and other cell types contain 2 major histone phosphokinase activities able to phosphorylate F1 (Lysine-rich) histone. These two activities designated KI and KII, were readily separated by G-200 gel filtration. KI, which is similar to the ubiquitous cyclic 3',5'-adenosine monophosphate (cAMP) - stimulated phosphokinase of all eukaryotic tissues, differs from KII by several criteria. KII is $\sim$90,000 M.W., cAMP-independent, rapidly turned over in vivo, low Km for ATP, and phosphorylates F1-histone at several unique sites.

Comparative examination of metaphase arrested (M) and counterpart interphase (I) cells for these two activities reveals that the KII enzyme is responsible for the overall high activity in M-arrested cells. Pulse labeling of cells with $^{32}P$ during traverse of the G2-M phase of the cell cycle reveals an in vivo tryptic phosphopeptide pattern unique to M cell F1 histone. Seven major phosphopeptides derived by in vitro phosphorylation of F1 with the KII enzyme correspond to these M cell specific phosphorylation sites observed in vivo. It can be concluded that KII activity predominates during the G2-M transition and that F1 is its natural in vivo substrate.

The phospho form of F1 that exists at metaphase due to the action of the KII phosphokinase is preserved until cells are allowed to enter G1. This is despite an in vivo decay of KII activity with long metaphase arrest times.

Significance to Bio-Medical-Research

Knowledge of structural and functional associations between the elements of chromatin in metaphase chromosomes is basic to an understanding of many cytogenetic observations. These include chromosome banding, virus-induced abberrations, and gene-activity relationships. Recognition of a distinct phospho-to dephospho transition of one of the major histone proteins at the G2-M boundary is likely to contribute ultimately to an understanding of the biochemistry of chromosome structure.

Proposed Course of Project:

This project will not continue when Dr. Lake leaves the laboratory on July 1, 1973.

Publications:

R. S. Lake and N. P. Salzman. Occurrence and properties of a chromatin-associated F1-histone phosphokinase in mitotic Chinese hamster cells. Biochemistry 11, 4817-4826. 1972.

Thomas Y. Shih and Robert S. Lake. Studies on the structure of metaphase and interphase chromatin of Chinese hamster cells by circular dichroism and thermal denaturation. Biochemistry 11, 4811-4817. 1972.

Robert S. Lake. F1-histone phosphorylation in metaphase chromosomes of culturec Chinese hamster cells. Nature New Biology 242, 145-146. 1973.

Robert S. Lake. Further characterization of the F1-histone phosphokinase of metaphase-arrested animal cells. J. Cell Biology. In press.

Serial No. NIAID-84C
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mechanism of DNA Replication and Integration

Previous Serial No.: Same

Principal Investigators: Dr. Norman P. Salzman
Dr. George C. Fareed
Dr. Alfred G. Kasselberg
Dr. Edwin D. Sebring
Ms. Marilyn M. Thoren
Mr. Michael Radonovich

Other Investigators: None

Cooperating Units: Dr. Jacob Lebowitz

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 90/12 | | 12/12 | 102/12 |
| Professional: | 60/12 | | 12/12 | 72/12 |
| Other: | 30/12 | | | 30/12 |

Project Description:

Objectives:

To define in molecular terms the mechanism of SV40 DNA replication and the process by which SV40 DNA becomes integrated into the cell genome and to understand mechanisms which control expression of the integrated viral genome.

Methods Employed:

SV40 infected cells have been fractionated to effect a separation of cellular and viral DNA. Radioactive precursors have been incorporated into replicative intermediates (RI's) of SV40. RI has been examined by electron microscopy, isopycnic centrifugation, and velocity rate sedimentation in

neutral and alkaline conditions. Immunofluorescent tests have been used to detect the T and V antigens of SV40.

Major Findings:

The mechanism of replication of SV40 DNA has been further defined. In previous studies we were able to show that DNA synthesis occurred at a specific site in the covalently closed duplex molecule. This finding has been confirmed by cleaving replicating SV40 DNA molecules with a bacterial restriction endonuclease. Examination of the cleaved molecules by electron microscopy has confirmed that replication is initiated at a specific site and further, has demonstrated that replication is bi-directional, that is there are two replicating forks that both move at the same rate in opposite directions. In a separate series of experiments the termination site for DNA replication has been located and is 50% of the genome length from the DNA initiation site. This finding is in complete agreement with bidirectional DNA replication.

A second series of experiments have defined the mechanism of chain growth during SV40 replication. Chain growth occurs by a discontinuous mechanism in which short 4S DNA fragments are made and then joined to growing DNA chains. These DNA fragments have been isolated, and they are able to reanneal to form duplex structures. This establishes that the two growing chains at each replication fork are made in a discontinuous manner. Inhibitor studies indicate that two DNA polymerases may be involved in chain growth. The first synthesizes the 4S fragment while the second must fill in a gap between the 4S fragment and the growing chain before it is joined by the action of ligase.

A third series of studies have been concerned with the mechanism of effecting semi-conservative replication of a covalently closed duplex. These studies have demonstrated (1) a decrease in the topological winding number which accompanies replication and, (2) evidence for a denatured region about twenty nucleotides in length at each of the replication forks.

Significance to Bio-medical Research and the Program of the Institute:

Integration of viral DNA into the cell genome is likely to occur with a number of viruses and is probably important in latent viral infection. These studies hope to explain viral latency and also to ellucidate critical steps in viral transformation by oncogenic viruses.

Proposed Course of the Project:

The role of viral and cellular enzymes in SV40 DNA replication will be defined. The mechanism of initiation of DNA replication will be defined.

Honors and Awards:

Editor, Journal of Virology

Member, Virology Study Section

Invited lectures at:

1. University of Geneva, Geneva, Switzerland.
2. Medical Research Council, Mill Hill, England.
3. Imperial Cancer Research, London, England.
4. Duke University, Durham, N. C.
5. ICN-UCLA Symposium on Molecular
6. Conference on the Eukaryotic Chromosome, Boulder, Colo.
7. University of Tennessee, Knoxville, Tenn.
8. Symposium on Chromosome Structure and Function, Cold Spring Harbor, N. Y.

Publications:

Fareed, G. C., Sebring, E. and Salzman, N. P.: Cleavage of replicative intermediates of simian virus 40 deoxyribonucleic acid by the restriction endonuclease of escherichia coli B. J. Biol. Chem. 247: 5872-5879, 1972.

Thoren, M. M., Sebring, E. D. and Salzman, N. P.: A specific initiation site for simian virus 40 deoxyribonucleic replication. J. Virol. 10: 462-468, 1972.

Fareed, G. C., Garon, D. F. and Salzman, N. P.: The origin and direction of SV40 DNA replication. J. Virol. 10: 484-491, 1972.

Fareed, G. C. and Salzman, N. P.: An intermediate in SV40 DNA chain growth. Nature New Biol. 238: 274-277, 1972.

Fareed, G. C., McKerlie, L. and Salzman, N. P.: Characterization of simian virus 40 DNA component II during viral DNA replication. J. Mol. Biol. 74: 95-111, 1973.

Salzman, N. P. and Thoren, M. M.: Inhibition in the joining of DNA intermediates to growing SV40 chains. J. Virol. In press.

Fareed, G. C., Khoury, G. and Salzman, N. P.: Self-annealing of 4S strands from replicating SV40 DNA. J. Mol. Biol. In press.

Salzman, N. P., Sebring, E. D. and Radonovich, M.: Unwinding of parental strands during SV40 DNA replication. J. Virol. In press.

Serial No. NIAID-85A
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structure of SV40 DNA

Previous Serial Number: Same

Principal Investigators: Dr. Frank DeFilippes

Other Investigators: None

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 16/12 | | | 16/12 |
| Professional: | 12/12 | | | 12/12 |
| Other: | 4/12 | | | 4/12 |

Project Description:

Objectives:

A) Characterization of a restriction enzyme from Hemophilus parainfluenza.

B) Use of this enzyme to produce defined nucleotide sequences of SV40 DNA.

Methods Employed:

A) The enzyme was purified by standard biochemical techniques. SDS-gel electrophoresis was used to obtain protein patterns after each purification procedure.

B) Definite double stranded sequences of SV40 DNA are produced by cleavages of the parainfluenza enzyme. These pieces can be separated and recovered by gel electrophoresis. The different pieces can themselves be continuously shortened in length by exonucleases and the different lengths can be separated and recovered by gel electrophoresis. The pieces are hybridized to viral specific RNA made at different times during the infectious cycle.

Major Findings:

A) Purification of the Hemophilus parainfluenza restriction enzyme:

Purification of the enzyme was facilitated by development of a rapid assay which measures the conversion of closed circular, duplex DNA to a cleaved form which can be detected by differential adsorption to hydroxyapatite after denaturation. After removal of nucleic acids in crude extracts by streptomycin precipitation, enzymatic activity is found in the 30-50% ammonium sulfate fraction. Phosphocellulose chromatography at pH 6.6 and then at pH 7.4 provides a 25 fold purification of the enzyme and removes a potent exonuclease activity found in the ammonium sulfate fraction. Sephadex G100 chromatography in 1M NaCl provides another 12-fold purification. The enzyme can be further purified by DEAE chromatography at pH 7.4 but the recovery is poor.

B) Characteristics of the enzyme:

The enzyme requires $Mg^{++}$ and mercaptoethanol. It operates efficiently over a wide range of NaCl concentrations and it appears to be stable at 37° in 0.5M NaCl pH 7.4 for a period of at least 5 hours. On Sephadex G100, in 1M NaCl, enzymatic activity elutes near an ovalbumin marker (MW 43,000). In 10mM-Na phosphate pH 7.4, the enzyme aggregates and elutes in the void volume of Sephadex G200. The aggregation does not affect enzyme activity.

1 microgram of enzyme protein from the phosphocellulose fraction will produce complete cleavage of 5 micrograms of SV40 DNA in 2 hours. However, the phosphocellulose fraction shows at least 16 protein bands by SDS-acrylamide gel electrophoresis.

C) Cleavage of SV40 virus DNA by the enzyme:

SV40 DNA is cleaved into 3 major fragments representing 43, 35 and 20% of the original DNA. These pieces are easily separated on 1.5% agarose gels from which they are eluted and further digested with exonuclease III or the exonuclease associated with T4 DNA polymerase. The exonuclease treated pieces can be sized on acrylamide-agarose gels so that different length single stranded DNA pieces with the original 5' end of the restriction enzyme fragments can be obtained. These pieces, of any desired length, can be used in liquid hybridization experiments.

SV DNA I was denatured at pH 13.0 and renatured by several procedures. Renaturation was determined by electrophoretic mobility and susceptibility to restriction enzyme cleavage. Dialysis against a low ionic strength buffer at neutral pH renatures most of the DNA. This procedure is as effective as more complicated schemes involving renaturation at high pH and elevated temperatures.

Significance to Bio-medical Research and the Program of the Institute:

Production of defined pieces of SV40 DNA may lead to the structure of the genetic element involved in the malignant transformation of cells.

Proposed Course of the Project:

Restriction enzymes will be used to produce unique fragments of SV40 DNA. These pieces will be continuously decreased in length by exonucleases. Pieces of any length will be used in liquid hybridization experiments with SV40 specific RNA.

Publications:

DeFilippes, F.: A simple assay for DNA restriction endonucleases. Anal. Biochem. 52: 637, 1973.

Serial No. NIAID-87A
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Properties of the Nucleic Acid and Protein Components of Adenovirus-Associated Viruses

Previous Serial Number: Same

Principal Investigator: Dr. James A. Rose

Other Investigators: Dr. Barrie J. Carter
Dr. George C. Lavelle
Mr. Frank Koczot
Mr. James Garrison

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 70/12 | | | 70/12 |
| Professional: | 36/12 | | | 36/12 |
| Other: | 34/12 | | | 34/12 |

Project Description:

Adenovirus-associated viruses (AAV) are small, DNA-containing viruses that multiply only in cells which are co-infected with an adenovirus. Herpesviruses also provide some function(s) required for AAV multiplication; AAV antigen(s) is synthesized but an increase in AAV infectivity or particles has not been observed, Four AAV serotypes are now known. We have shown previously that AAV DNA is segregated in a way not as yet observed with any other virus. Single, complementary DNA strands are separately encapsidated in different virions. On extraction of viral DNA, the complementary strands anneal to form duplex molecules. There are two species of complementary DNA strands which can be separated in CsCl density gradients. Studies are directed toward the biological and biochemical characterization of AAV infection.

Objectives:

1. To analyze the structure and function of the complementary strand species of AAV-DNA.

2. To identify the helper virus-mediated step(s) in the AAV replication sequence.

3. To analyze the structure and function of *in vivo* synthesized AAV RNA species induced by either partial or complete helper viruses.

Methods Employed:

The AAV are produced in KB cells in spinner culture. Viruses are purified in CsCl density gradients, and DNA is extracted from purified virus by treatment with alkali or with proteolytic enzymes, sodium dodecyl sulfate and phenol. The complementary strands species are separated by centrifuging denatured, bromodeoxyuridine-substituted DNA to equilibrium in neutral CsCl density gradients. Viral RNA synthesized *in vivo* or *in vitro* is labeled with $^{3}H$ or $^{32}P$. DNA/RNA complementarity is determined by nucleic acid hybridization and hybrids are detected on nitrocellulose filters, in CsCl density gradients or by hydroxyapatite chromatography. RNA is further analyzed by sedimentation in dimethyl-sulfoxide sucrose gradients. DNA molecules are directly visualized in the electron microscope using either aqueous or formamide spreading techniques.

Major Findings:

1. Nucleic acid hybridization procedures were used to measure the extent of transcription of adenovirus-associated virus (AAV) deoxyribonucleic acid (DNA) in KB cells in the presence of either adenovirus or herpes simplex virus as the helper. Annealing of AAV ribonucleic acid to AAV DNA was monitored by a hybridization inhibition assay on nitrocellulose filters or by hydroxyapatite chromatography. These experiments confirmed the previous observation that, in the presence of either type of helper virus, only one strand of AAV DNA (the thymidine-rich or "minus" strand) is transcribed in vivo. However, it was found that only 70 to 80% of this strand appears to be transcribed in vivo. Furthermore, studies with minus strands employing hydroxyapatite chromatography and nuclease $S_1$, which specifically degrades single-stranded DNA, indicated that up to 20% of the minus strand may be self-complementary. It seems likely that these self-complementary sequences account for the bulk of that portion of the minus strand (20 to 30%) which is not transcribed in vivo.

2. At least 70% of plus or minus strands of adenovirus-associated virus DNA contain self-complementary sequences at or near their termini. Self-annealing of these sequences generates circular molecules that are closed by duplex, hydrogen-bonded segments. The self-annealed segments are sensitive to exonuclease III and have a thermal stability comparable to that of double-stranded DNA molecules. Length measurements of double-stranded adenovirus-associated virus DNA molecules

show a bimodal distribution, with the larger component being 10% shorter than SV40 DNA.

The presence of self-complementary terminal sequences in single-stranded molecules of viral DNA has been observed previously only with DNA from adenoviruses. It is thus especially notable that adenovirus-associated virus replication is unconditionally dependent on a helper adenovirus. The terminal self-complementary sequences could play a role in viral DNA replication.

3. The temporal relationships of viral DNA and RNA synthesis in KB cells co-infected with adenovirus-associated virus type 2 (AAV-2) and adenovirus type 2 (Ad2) as the helper have been analyzed. In cells simultaneously infected with AAV-2 and Ad2 both AAV and Ad DNA synthesis began at 6.5 to 7.5 hours. AAV RNA synthesis began at 9-10 hours whereas Ad RNA synthesis could be detected at 2-3 hours and increased greatly in rate at 10 hours. When cells were infected with Ad2 for 10 hours prior to infection with AAV-2, the interval between AAV DNA and RNA was abolished and synthesis of both began within 3 to 4 hours after AAV infection. Furthermore, in a simultaneous infection AAV transcription did not occur when viral DNA synthesis was blocked with fluorodeoxyuridine (FUDR). These findings suggest that (i) AAV DNA and RNA synthesis require separate helper functions which appear sequentially after Ad infection, (ii) both AAV and Ad DNA synthesis may be initiated by the same factor(s), (iii) the initiation of AAV RNA synthesis may depend upon a factor which controls late adenovirus transcription, and (iv) initiation of AAV RNA synthesis requires viral DNA synthesis.

Significance to Bio-medical Research and the Program of the Institute:

Human infection with AAV has been shown to be frequent, but its significance is not yet known, since a virus-specific clinical disease has not been defined. Characterization of biochemical and biological properties of these viruses may be important with respect to human disease, because 1) although now inapparent, they might be human pathogens and, 2) the mechanism(s) by which helper viruses assist AAV replication are relevant to understanding natural modes of viral infection and may be important with respect to the development of biochemical controls of virus replication. In contrast to cell DNA, the relatively small genomes of these viruses permit more comprehensive biochemical and physical analyses of function and structure. For example, during the past year we were the first to identify and define a new type of genetic structure at the ends of both the adenovirus and AAV genomes (inverted terminal repetition). These terminal sequences may explain the mechanism of DNA synthesis for both viruses as well as represent (at least partially) the molecular reason for AAV defectiveness. Because this terminal structure appears to be highly specific, further definition of its biochemical properties could provide an important basis for identifying or preparing compounds with selective antiviral activity. In addition, studies on the biochemical dependence by AAV for specific adenovirus gene products indicate that this

system may be used to purify and characterize these control substance. Such information could provide insight into basic properties of gene regulation in general as well as offer new approaches in the development of antiviral agents.

Proposed Course of the Project:

Continue to study AAV replication and defectiveness.

Honors and Awards: None

Publications:

Rose, J. A. and Koczot, F.: Adenovirus-associated virus multiplication VII. Helper requirement for viral DNA and RNA synthesis. J. Virol. 10: 1-8, 1972.

Carter, B. J. and Rose, J. A.: Adenovirus-associated virus multiplication VIII. Analysis of in vivo transcription induced by complete or partial helper viruses. J. Virol. 10: 9-16, 1972.

Carter, B. J., Khoury, G. and Rose, J. A.: Adenovirus-associated virus multiplication IX. The extent of transcription of the viral genome in vivo. J. Virol. 10: 1118-1125, 1972.

Koczot, F. K., Carter, B. J., Garon, C. and Rose, J. A.: Self-complementarity of terminal sequences within plus or minus strands of adenovirus-associated virus DNA. Proc. Nat. Acad. Sci. 70: 215-219, 1973.

Carter, B. J., Koczot, F. K., Garrison, J., Dolin, R. and Rose, J. A.: Adenovirus-associated virus multiplication: Separate helper functions provided by adenovirus. Nature. In press.

Serial No. NIAID-87B
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Properties of Adenovirus DNA

Previous Serial No.: Same

Principal Investigators: Dr. Claude Garon
Dr. James A. Rose

Other Investigators: Ms. K. W. Berry
Mr. James Garrison

Cooperating Units: Dr. John C. Hierholzer, Respiratory Virology Section, Center for Disease Control, Atlanta, Ga.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 24/12 | | | 24/12 |
| Professional: | 12/12 | | | 12/12 |
| Other: | 12/12 | | | 12/12 |

Project Description:

Objectives:

To provide a detailed map of base sequence homologies and heterologies among the genomes of several oncogenic and nononcogenic adenovirus groups. And, further, to correlate mappable sequences with a biochemical characterization of both structural and replicative properties of these viruses.

Methods Employed:

Viruses are produced in KB cells and purified in CsCl equilibrium density gradients. Viral DNA is extracted from purified virus by treatment with proteolytic enzymes, sodium dodecylsulfate, and phenol. The electron microscope heteroduplex method consists of constructing in vitro a molecule in which one strand of the DNA duplex is derived from each of the viruses being

compared. Using the formamide modification of the basic protein film procedure for visualization of DNA, regions of sufficient homology form duplex structures, while regions containing heterologous sequences remain as recognizably unpaired single strands. Careful measurement of these structural features reveal both the extent and map location of sequence heterology between the viruses being compared. Heteroduplexes may be effectively purified using benzoylated-naphthoylated-DEAE-cellulose (BND) column chromatography and specifically with single strand specific nucleases isolated from neurospera crassa.

Major Findings:

When DNA from adenovirus serotypes was mixed, denatured and renatured characteristic heteroduplex molecules were formed. These molecules, composed of one strand from one serotype and one strand from the other serotype, contained one or more detectable regions of strand separations (or heterology) depending upon denaturing conditions and the combination of type specific DNA preparations. By increasing denaturing conditions, regions of partial homology could be demonstrated in all interserotypic heteroduplexes. The heteroduplex patterns in heteroduplexes constructed with DNA from nononcogenic serotypes (Ad 1, 2 and 5) were relatively simple and nearly similar, whereas both simple and complex patterns were seen in heteroduplexes prepared with DNA from weakly oncogenic serotypes (Ad 3, 7 and 21). Only complex heterology patterns were found in heteroduplexes prepared with DNA from highly oncogenic serotypes (Ad 12, 18 and 31). As expected, the most extensive heterologies were observed in heteroduplexes prepared with DNA from serotypes belonging to different serologic groups. Within groups, heteroduplex molecules contained two specific regions in which heterologies were most marked. These regions appeared to map at similar locations among heteroduplexes from all groups. Furthermore, it was found that the integrated SV40 virus DNA in an Ad7-SV40 hybrid genome was situated in one of these regions.

Heteroduplex molecules could be clearly separated from homoduplexes using a benzoylated-naphthoylated-DEAE-cellulose (BND) column. Homoduplexes were eluted from the column in 1.0 M NaCl while molecules containing areas of mismatch were effectively eluted with 2% caffeine. Treatment of the heteroduplex fraction with single-strand specific nucleases (Neurospera crassa) produced fragments which correspond in size to the double-stranded regions of the intact heteroduplex molecules. No fragmentation was observed in similarly treated native or homoduplex samples. A similar fractionation was obtained with heteroduplexes between DNA from prototype and non-prototype strains of Ad 5 or Ad 3. These molecules contained no visible areas of mismatch. It therefore appears that Neurospera nucleases can detect mismatched regions in heteroduplex molecules which are too small to be seen in the electron microscope.

Significance to Bio-medical Research and the Program of the Institute:

The added dimension provided by the electron microscope heteroduplex mapping method over DNA-DNA hybridization techniques is the ability to localize (possibly within several nucleotide bases using techniques developed in this laboratory) regions of homology and non-homology along the contour length of an intact molecule. Furthermore, the treatment of purified heteroduplex molecules with single-strand specific nucleases provides a procedure for generating quantities of both physically and genetically defined fragments that are of great value in mapping specific loci in related viral genomes.

Proposed Course of the Project:

(1) to continue using the electron microscope heteroduplex mapping technique to provide direct structural characterizations of members of several oncogenic and nononcogenic adenovirus groups.

(2) To use these data and that already obtained by these methods to correlate genetic maps with biochemical functions expressed during viral infection.

Honors and Awards:

None

Publications:

Garon, C. F., Berry, K. W. and Rose, J. A., 1972. A unique form of terminal redundancy in adenovirus DNA molecules. Proc. Natl. Acad. Sci., 69: 2391-2395.

Garon, C. F., Berry, K. W., Hierhalzer, J. C., and Rose, J. A. Mapping of base sequence heterologies between genomes from different adenovirus serotypes. Virology, in press.

Serial No. NIAID-88
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Synthesis of Proteins in SV40 Infected Cells

Previous Serial No: Same

Principal Investigator: Dr. Stanley Barban

Other Investigators: Mr. Henry Schulze (Thru Nov. 1972)

Cooperating Units: Dr. Ettore Appella, N.C.I.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned To Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 15/12 | | | 15/12 |
| Professional: | 12/12 | | | 12/12 |
| Other: | 3/12 | | | 3/12 |

Project Description:

Objectives:

To elucidate the role of the viral genome in directing the synthesis of both viral and non-structural proteins in productive and transforming infection.

To analyze the peptide and amino acid composition of the capsid proteins of SV40 mutants.

To examine and characterize the proteins that are associated with the SV40-viral DNA in infected and transformed cells.

Methods Employed:

Virus was grown in primary and continuous African green monkey kidney cells

and purified by density gradient sedimentation. Fractionation of the labeled proteins and subunits of purified virions was performed by gel electrophoresis, column chromatography, isoelectric focusing, enzymatic digestion, peptide mapping by thin layer chromatography, and amino acid analyses. Specific viral proteins were further analyzed by immunological methods.

Major Findings:

Electrophoretic differences in the capsid proteins of SV40 plaque mutants - We have previously shown that it was possible to distinguish large and small plaque mutants of SV40 by the isoelectric focusing technique. Further characterization of the capsid proteins of the mutants by this technique revealed a difference of 0.2 pH unit. The capsid protein of the small plaque mutant had an isoelectric point of pH 6.51 as compared with pH 6.28 for the large plaque strain. It was also found that the SV40 coat protein was heterogenous consisting of a major and two minor subunits.

Peptide and amino acid analyses of the capsid proteins - An explanation for the difference in electrophoretic charge in the capsid proteins of small and large plaque SV40 mutants was obtained by peptide analyses. A single peptide difference was found in the peptide maps obtained from the tryptic digests of the capsid proteins of the SV40 mutants after separation by electrophoresis on thin layer and detection by autoradiography.

Subunit composition of the SV40 capsid protein. Examination of the number of peptides obtained by fingerprinting of SV40 capsid protein reveals only half the number of spots predicted from amino acid analyses of the lysine and arginine residues. These results suggest that the SV40 capsid protein is composed of two identical subunits.

Significance to Bio-medical Research and the Program of the Institute:

The objective of these studies is a determination and characterization of the polypeptides specified by the SV40 viral genome and those involved in maintaining the transformed cell phenotype of non-permissive cells transformed by SV40 The ultimate goal is, of course, the isolation and identification of the transforming gene product. These studies have obvious basic and practical significance to problems of control mechanisms of virus induced host cell alterations.

Proposed Course of the Project:

The nature of the subunit structure of the capsid protein will be further examined by specific amino acid labeling and fingerprint analyses.

Studies of the proteins and polypeptides found in association with the viral DNA in lytic and transformed infections will be continued.

Honors and Awards:

None

Publications:

Barban, S.: Electrophoretic Differences in the Capsid Proteins of Simian Virus 40 Plaque Mutants. Journal of Virology, in press.

Serial No. NIAID-88B
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Molecular Biology of Chromatin and Regulation of Viral Gene Activity in Cell Transformation

Previous Serial Number: Same

Principal Investigators: Dr. Thomas Y. Shih
Dr. Malcolm A. Martin
Dr. George Khoury
Ms. Janet Byrne

Other Investigator: Dr. Robert S. Lake

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 26/12 | | | 26/12 |
| Professional: | 18/12 | | | 18/12 |
| Other: | 8/12 | | | 8/12 |

Project Description:

Objectives:

To study the molecular structure and biochemical activities of isolated chromatin and their relationship to the regulation of viral gene expression in the process of cell transformation.

Methods Employed:

A. A procedure was developed for the isolation of chromatin from transformed cells in tissue culture involving the homogenization of cell suspension in saline-EDTA and differential centrifugation. The chromatin thus isolated was characterized for chemical composition, thermal melting profiles and circular dichroism spectra. Its activity as a template for RNA synthesis compared to purified DNA, was assayed by the addition of exogenous DNA-dependent RNA polymerase. Separated strands of $^{32}P$ SV40 DNA fragments were used as a molecular probe to study the viral specific RNA transcribed from chromatin in vitro.

to purified DNA, was assayed by the addition of exogenous DNA-dependent RNA polymerase. Separated strands of $^{32}$P-SV40 DNA fragments were used as a molecular probe to study the viral specific RNA transcribed from chromatin *in vitro*.

B. In order to more fully understand the regulation of the integrated viral genome in transformed cells, a general method of gene isolation was developed to isolate the viral DNA sequences from transformed cells. The method involves chemical linkage of SV40 cRNA prepared *in vitro*, to a cellulose matrix by a water soluble carbodiimide and continuous circulation of DNA containing specific sequences complementary to the RNA.

Major Findings:

A. It has previously been shown that chromatin is a restricted template; only a small portion of the chromatin DNA is available for transcription. Studies on the template activity of partially dehistonized chromatins and reassociated DNA-histone complexes have indicated that histone is the major repressor substance. When purified chromatin isolated from a SV40 transformed mouse cell line (11A8) was examined, the template activity is about 17% that of purified DNA confirming that more than 80% of chromatin DNA is repressed presumably by its association with chromosomal proteins. As a step toward determining the nature of repressor substance responsible for implied partial or complete repression of integrated viral genomes, SV40 DNA was employed as a molecular probe to study the viral specific RNA transcribed by *E. coli* RNA polymerase from chromatin of SV40-transformed cells. The presence of SV40 specific RNA sequences was detected by annealing the RNA with separated strands of $^{32}$P-SV40 DNA fragments in solution and the hybrid formed was analyzed by hydroxyapatite chromatography. The RNA transcript of the integrated SV40 genome appears to be assymmetric; it hybridized to a much greater extent to the minus than to the plus strand of SV40 DNA. This is similar to the pattern observed *in vivo*. The viral specific RNA also shares sequences with "early" lytic SV40 specific RNA, and is, in general, similar to that present in SV40-transformed cells.

B. If the functioning genes of a given cell are regulated by the proteins associated with chromosomal DNA, the restriction imposed on chromatin DNA should be transmitted quite faithfully to daughter cells. This question was examined by studying changes of DNA-histone interaction and nucleohistone structure in the process of mitosis as manifested by condensation of chromosomes at the metaphase. Chromatins were isolated from metaphase Chinese hamster cells arrested by vinblastine as well as the remaining interphase cells. The structure of nucleohistone was studied by thermal denaturation profiles and circular dichroism spectroscopy. The results indicate that the elementary structure of nucleohistone, such as the histone stabilization of DNA double helix and supercoiled configuration of the nucleohistone fiber, is preserved during mitosis, thereby ensuring that the same chromatin structure is passed to daughter cells.

C. Another step was taken to isolate the integrated viral genome from transformed cell DNA. SV40 cRNA, assymmetrically transcribed from SV40 DNA I by E. coli RNA polymerase, was chemically linked to a cellulose matrix by N-Cyclohexyl-N'-B-(4-methylmorpholinium) ethyl carbodiimide p-toluenesulfonate. The SV40 RNA-cellulose was then packed in the reassociation chamber portion of a continuous hybridization system. The cellulose matrix was maintained at 37°C (50% formamide, 2XSSC) to allow efficient DNA-RNA interaction in the stationary phase, while unreacted DNA and any reassociated DNA in the mobile phase was denatured at 90°C and then recirculated into the hybridization chamber. Between 40 and 45% of fragmented $^{32}$P-SV40 DNA (very close to the theoretical 50%) was removed from the circulating solution. In the presence of $10^4$-fold excess of sheared E. coli DNA, the same amount of $^{32}$P-SV40 DNA was isolated with negligible contamination by the unrelated bacterial DNA. The efficiency and selectivity of this method will permit the isolation of integrated SV40 DNA sequences from virus-transformed cells.

Significance to Bio-medical Research and the Program of the Institute:

SV40 DNA provides a useful molecular probe to study the regulation of gene expression in eukaryotic chromatin in cells transformed by oncogenic viruses. Study of the transcriptional properties of chromatin may ultimately lead to the identification of the repressor molecules which regulate the expression of endogeneous viral genetic information. Development of a method for specific gene (DNA sequence) isolation may yield more information on how the integrated viral gene activity is regulated. The ultimate goal of these efforts is to understand how regulation of the genotype leads to the various phenotypes of a cell and thereby provides rational basis for development of therapeutic agents for modifying the regulatory mechanisms.

Proposed Course of the Project:

The detailed molecular pattern of the SV40 specific RNA transcribed from chromatin prepared from virus transformed cells will be further characterized by RNA-DNA hybridization. A method to concentrate the small amounts of viral specific RNA present in transformed cells is presently being developed. The method of gene isolation will be further refined in order to isolate and characterize the SV40 DNA sequence from virus-transformed cells.

Honors and Awards:

Invited lecture in course on Protein-Nucleic Acid Interactions in the NIH Graduate Program.

Publications:

Shih, T. Y. and Lake, R. S.: Studies on the Structure of metaphase and interphase chromatin of Chinese hamster cells by circular dichroism and thermal

denaturation. Biochemistry, 11, 4811-4817 (1972).

Shih, T. Y. and Martin, M. A.: A General Method of Gene Isolation. Proc. Nat. Acad. Sci., in press, June issue, 1973.

LABORATORY OF MICROBIAL IMMUNITY


Summary . . . . . 1

110 - Administration, Research Planning and Coordination . . . . . 5
111-B - Studies on the Pathogenesis of Amebic Enteritis. . . . . 6
111-C - Investigations on Blastocystis hominis in Germfree and Controlled-Flora Animals. . . . . 8
111-E - Studies on Diffuse Amebic Hepatitis with Histochemical Study of the Liver . . . . . 10
111-F - Histochemistry of the Intestine in Acute Amebic Enteritis. . 12
111-G - The Pathogenic Determinants in Entamoeba histolytica with Special Reference to Intracellular Viruses of Non-Pathogenic and Highly Pathogenic Strains. . . . . 14
111-H - Primary Amebic Meningoencephalitis Due to Free-Living Amebas of the Genus Naegleria . . . . . 16
113 - The Genetic, Cellular and Regulatory Factors in Autoallergic Disease. . . . . 18
113-E - Immunologic Tolerance. . . . . 22
116 - Control of Immunoglobulin Synthesis. . . . . 26
116-J - The Suppressive Effects of Heterologous Antithymocyte Sera on the Homograft Reactions in Mice . . . . . 30
117 - The Immunologic Response of Conventional and Germfree Mice to Blood-Induced Infections of Plasmodium berghei. . . . . 32
117-C - Separation of Different Erythrocytic Stages of Plasmodium berghei and P. knowlesi. . . . . 34
117-E - The Immunologic Response of Animals Immunized With Trypanosomes . . . . . 36
118 - Synergy in the Graft-versus-Host Response. . . . . 38
119-A - Quantitative and Qualitative Studies on the Antibody Response to Pneumococcal Polysaccharides at the Cellular Level 42

PHS-NIH
Summary Statement
Laboratory of Microbial Immunity
July 1, 1972 through June 30, 1973

Studies in this Laboratory concern primarily the basic mechanisms involved in cellular and humoral immune responses to microbial and tissue antigens. A special facility is maintained for work in which germfree animals or animals with a controlled flora are needed.

There has been little change in staff this year. Dr. Robert E. Tigelaar is on working assignment at University College, London, England, and is expected to return in October. His work in England has mostly involved the study of interaction among thymus-derived (T) lymphocytes *in vitro* and *in vitro* assays of T lymphocyte function. The appointment of Dr. Vilho Pasanen, a Visiting Fellow, was renewed for one year. Dr. David R. Barthold will complete a very productive two years as a Staff Associate. Dr. Herbert C. Morse, III, joined the Laboratory as a Research Associate in July, 1972. He is extending the work of previous associates on cell interactions in graft-*versus*-host reactions. Each of the Laboratory's programs has made significant progress, as indicated in the research accomplishments.

SUPPRESSOR "T" CELLS REGULATE THE PROLIFERATION OF LYMPHOCYTES

The magnitude of the antibody response to Type III pneumococcal polysaccharide (SSS-III) is regulated by the activities of two types of thymic-derived cells (T cells) having opposing functions; these have been termed amplifier and suppressor T cells. The ability of antilymphocyte serum (ALS) to enhance the antibody response to SSS-III is apparently due to the inactivation of suppressor cells which, in contrast to amplifier cells, exert a negative effect upon the magnitude of the antibody response normally produced following immunization. Current studies show that suppressor T cells act primarily by limiting the extent to which antibody-producing "B" cells proliferate in immunized mice; other investigators have shown that suppressor T cells can also effect the antigen-induced proliferative response of other T cells in a similar manner. Suppressor T cells may provide a homeostatic control mechanism for limiting the clone size of all types of lymphoid cells and thereby play an important role in preventing the development of lymphoproliferative disorders and autoimmune disease. (Dr. Baker, Mr. Stashak, and Miss Amsbaugh, with Dr. Prescott, Laboratory of Microbiology, NIAID)

DISSOCIATION OF DELAYED HYPERSENSITIVITY TO THYROID AND UVEA AND AUTOIMMUNE DISEASE

Experimental allergic thyroiditis may be produced in guinea pigs by immunization with thyroid extract and complete Freund's adjuvant. The relation between delayed type hypersensitivity and the onset, severity and persistence of the disease was followed by skin testing the animals at appropriate times. The delayed reaction appeared just before the onset of disease, and persisted for several months, reaching maximum at 2-3 months simultaneously with the maximum disease severity. However, it did not last as long as the disease, for the disease lasted two years, but the delayed sensitivity only 3-6 months. In further studies, an attempt to produce delayed sensitivity without disease was made. To do this animals were immunized with bovine gamma globulin (BGG) and thyroid together in Freund's adjuvant. In certain combinations mild delayed reactions to the thyroid appeared in the absence of thyroiditis. Similarly, attempts to produce delayed sensitivity to uveal extract without producing uveitis were undertaken. In these studies animals were injected with uvea and BGG. They were then reimmunized with uvea and BGG or uvea alone. At the end of the study they were skin tested and the results compared with controls immunized and reimmunized with uvea alone. All gave good delayed skin tests, 20-40 mm in diameter to uveal antigen. There was no apparent reduction in delayed reactivity as a consequence of adding BGG to the uveal immunizing antigen. However, those given BGG with the uvea did not develop uveitis even when reimmunized with sufficient adjuvant and uvea so as to produce uveitis de novo, thus adding BGG to an autoantigen may or may not reduce the degree of delayed type auto-allergy, but independently may prevent the disease. (Dr. McMaster)

PRIMARY AMEBIC MENINGOENCEPHALITIS PRODUCED IN GERMFREE GUINEA PIGS BY INTRANASAL INOCULATION WITH FREE-LIVING AMEBAS

Free-living amebas of the genus Naegleria, of world-wide distribution and long considered harmless, have been linked etiologically with 57 fatal cases of primary amebic meningo-

encephalitis during the last decade. The disease is unresponsive to drug therapy and there have been no known survivors of these infections. Naegleria from cultures derived from a fatal case of the disease in Richmond, Virginia, were inoculated intranasally, intraorally, into the conjunctival sac near the inner canthus of the eyes, and into induced skin lesions in adult germfree guinea pigs. Of 33 animals inoculated intranasally with 18-31 amebas, 31 developed a fatal encephalitis. None of the animals inoculated by the other 3 routes developed either symptoms or lesions. The studies confirm the ability of this ameba to penetrate the nasal mucosa and suggest that this is almost certainly its method of entering the host; all human cases have occurred in swimmers shortly after swimming in contaminated water. The presence of an intracellular diphtheroid symbiont growing in the ameba that had previously escaped detection by routine bacteriological procedures was revealed by growth of this organism in germfree hosts. This disease represents a potential public health problem of appreciable magnitude due to the ubiquitous distribution of the causative organism and to the fact that it is insensitive to as high as 10 p.p.m. chlorine. The germfree guinea pig is a very suitable host for study of the disease and for the testing of drugs for its chemotherapeusis. (Mr. Phillips)

ASSOCIATION OF DELAYED HYPERSENSITIVITY AND RESISTANCE TO T. RHODESIENSE

T. rhodesiense infections in man and animals stimulate the synthesis of large amounts of serum antibody. Since infected individuals usually succumb, this antibody is apparently not protective. Rabbits were infected with T. rhodesiense, an organism which usually causes death 8-10 weeks after infection, despite persistant high antibody titers. Six weeks later, some rabbits were injected with killed organisms. Serum antibody titers to the trypanosomes then decreased, and most animals recovered. Another group of rabbits were first immunized with killed organisms and six weeks later were challenged with live organisms. Animals that had no detectable serum antibody were resistant to challenge infections. When normal and infected animals were skin tested with an antigen prepared from live trypanosomes, all failed to give delayed skin reactions. Rabbits immunized with killed trypanosomes, either before or after infection, gave large delayed skin reactions. This work indicates that 1) serum antibody to T. rhodesiense has little effect in protecting the host, 2) cell-mediated immunity plays a major role in conferring resistance to trypanosomiasis, and 3) such resistance can be obtained by appropriate immunization after infection has been established. (Dr. Finerty and Mr. Evans)

DIRECT GRAFT-VERSUS-HOST REACTIVITY AND AMPLIFYING FUNCTION OF LYMPH NODE CELLS DISSOCIATED

The capacity of peripheral lymph node (LN) cells to produce graft-versus-host (GVH) reactions and their capacity to interact synergistically with thymocytes in producing GVH reactions has been demonstrated previously. LN cells were obtained from BALB/c mice which had been inoculated with anti-thymocyte serum at various intervals prior to sacrifice. Directly measured GVH activity was less than 10% of normal 2 and 11 days after treatment and was about 40% 30 days after treatment. LN cells obtained 2 days after treatment produced little or no synergistic interaction with thymocytes; LN cells obtained 11 days after treatment produced a much more active interaction with thymocytes (120-150% of normal); LN cells obtained at day 30 had about normal activity.

Preliminary studies indicate that this rapid recovery of "amplifying activity" will occur in the absence of a thymus. The data show a clear dissociation between directly measured GVH activity and amplifying activity, and support the hypothesis that these activities are mediated by separately differentiated lines of thymus-derived cells. (Drs. Morse, Tigelaar and Asofsky)

Serial No. NIAID - 110
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

**Project Title:** Administration, research planning and coordination

**Previous Serial Number:** Same

**Principal Investigator:** Richard Asofsky

**Other Investigators:** None

**Cooperating Units:** None

**Man Years:**

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 12/12 | | | 12/12 |
| Professional: | 2/12 | | | 2/12 |
| Other: | 10/12 | | | 10/12 |

**Project Description:**

Administrative and supervisory support is provided to all personnel and to each project of the Laboratory.

Research plans are discussed with the various investigators, over-all evaluations made and the research activities of the Laboratory coordinated. Collaborative research with specialists in other laboratories of NIAID, or institutes of the NIH, or various university investigators is encouraged and assistance given the professional staff in effecting the necessary arrangements. Technical consultation is provided to individuals of the NIH, and to scientists of other departments of the Government or academic organizations. Assistance is provided the professional staff in the preparation of manuscripts and in editing and reviewing a variety of technical reports. The needs of all personnel are evaluated and an effort made to obtain the necessary equipment and supplies for the efficient conduct of research. Budget requirements are forecast on a yearly basis and emergency needs dealt with as problems arise. Supervision is given in regard to laboratory expenditures, travel of personnel, and correspondence. When possible highly qualified professionals and technicians are sought as staff additions or replacements to insure a high level of research excellence.

Serial No. NIAID - 111-B
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the pathogenesis of amebic enteritis

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: Akio Takeuchi

Cooperating Units: Department of Experimental Pathology, Walter Reed Army Institute of Research, Washington, D.C.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 5/12 | | | 5/12 |
| Professional: | 4/12 | | | 4/12 |
| Other: | 1/12 | | | 1/12 |

Project Description:

Objectives:

To observe and record the microscopic alterations and events associated with the penetration of enteric tissues by Entamoeba histolytica including response of tissue including its microcirculation to amebic invasion.

Methods Employed:

Germfree guinea pigs are inoculated with E. histolytica and the accompanying enteric flora from acute cases of amebic enteritis in humans. At various intervals after inoculation the animal is anesthetized with ethyl carbamate and a newly-developed method for microscopic observation of the cecal mucosa and its microcirculation in the living anesthetized animal is employed for observing changes associated with pathogenesis of the disease. An ever present 16 mm movie camera is used to record tissue events which occur by time-lapse photomicrography.

Major Findings:

The first phase of the studies has been reported. This concerned the

mechanism(s) of tissue penetration by ameba. The current phase is new and much effort has gone into development of the complex technical procedures involved. Observations thus far have been limited to those involving early effects on the microcirculation with stagnation and thrombosis together with some increase in wandering histiocytes but no direct observations as yet of amebic penetration of epithelium.

Significance to Biomedical Research and the Program of the Institute:

Although we have previously observed and reported the first detailed study of mechanisms of tissue invasion in amebic enteritis based on large scale (EM) fine structure observations, it is important in this ubiquitous disease to learn as much as possible about the microscopic events associated with its pathogenesis.

Proposed Course of Project:

To continue along lines indicated above and to develop additional procedures as required to realize the stated objective.

Honors and Awards:

Bruce P. Phillips was elected to membership on the Board of Directors of the Association for Gnotobiotics.

Publications:

Takeuchi, A. and Phillips, B. P.: Electron microscope study of experimental amebiasis. Penetration of the colonic epithelium by Entamoeba histolytica. Proc. 69th Ann. Meeting Am. Assoc. Pathol. Bact., Am. J. Path. 66: 29a-30a, 1972.

Serial No. NIAID - 111-C
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Investigations on Blastocystis hominis in germfree and controlled-flora animals

Previous Serial Number: Same

Principal Investigator: Bruce P. Phillips

Other Investigators: Charles H. Zierdt

Cooperating Units: Lab. Clin. Pathol., Clinical Center, NIH, Bethesda, Maryland

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 2/12 | | | 2/12 |
| Professional: | 1/12 | | | 1/12 |
| Other: | 1/12 | | | 1/12 |

Project Description:

Objectives:

To ascertain whether Blastocystis hominis from symptomatic cases of enteritis possibly attributable to this organism does indeed have pathogenic potential and, if so, under what conditions.

Methods Employed:

Blastocystis hominis and its accompanying enteric flora from Clinical Center patients with enteric symptoms of undiagnosed etiology is introduced intracecally into germfree guinea pigs. Animals are observed for symptoms and are sacrificed at various intervals and examined for enteric tissue changes and for degree of B. hominis infection in the gut.

Major Findings:

We previously showed that B. hominis could be freed of its accompanying flora with a combination of 8 antibiotics but it would not survive in the gut of the germfree guinea pig. More recently, success has been achieved in cultivating this protozoan axenically in pre-reduced medium (it is an obligate an-

aerobe) and these organisms have likewise shown an inability to survive in the germfree gut (which has a high redox potential). The organism has also exhibited an inability to establish infections and grow in the intestine of conventional animals. However, recent experiments wherein B. hominis and its accompanying enteric flora from human patients were inoculated intracecally into germfree guinea pigs have produced many heavy infections. In several of these infections the animals have shown diarrheal symptoms and some weight loss. In one animal with a particularly heavy infection the cecum showed a superficial erythema.

Significance to Biomedical Research and the Program of the Institute:

There have been a number of hospital patients in whom severe enteric symptoms could not be attributed to the presence of any known pathogens but who harbored heavy infections with B. hominis. It is important to ascertain if this protozoan, long erroneously classified as a yeast and considered harmless, does possess pathogenic potential and if so under what conditions does it manifest pathogenicity.

Proposed Course of Project:

We plan to soon report these results which indicate that this organism is capable of eliciting symptoms under conditions of our experimentation and to continue to test additional strains as they become available and try to determine if this organism requires any particular associate in order to cause symptoms.

Honors and Awards: None

Publications: None

Serial No. NIAID - 111-E
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on diffuse amebic hepatitis with histochemical study of the liver

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: H. R. Jervis and Akio Takeuchi

Cooperating Units: Department of Experimental Pathology, Walter Reed Army Institute of Research, Washington, D.C.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 2/12 | | | 2/12 |
| Professional: | 1/12 | | | 1/12 |
| Other: | 1/12 | | | 1/12 |

Project Description:

Objectives:

To ascertain if acute amebic enteritis with amebas confined to enteric tissue elicits (as alleged) any alterations in the normal histology and histochemistry of the liver.

Methods Employed:

Acute amebic enteritis is induced in germfree guinea pigs by inoculating them intracecally with E. histolytica and the accompanying enteric flora from human cases of the disease. The livers of the animals, at various stages of the enteric infection, are studied by histological and histochemical procedures and compared with normal controls harboring only the bacterial flora from the aforementioned human cases.

Major Findings:

Animals with very severe enteric infections showed no or practically no glycogen in the liver whereas this was abundant in animals without or with

only minimal enteric amebic infection. The severely infected animals had large amounts of ORO+ material in the liver circulation with fat in the shape of small droplets lining the cell membrane of hepatocytes. In some instances, much larger fat inclusions were seen around the central vein. Less severely infected animals showed some fat in the hepatocytes but none in the liver circulation. Although fat in the liver was indicative of liver damage, this could not be supported by histological observation except in 1 of 34 guinea pigs examined; this animal had limited areas of liver necrosis not related to the presence of any amebas. G-6 Pase activity was higher in control than infected animals. In individual livers it was inversely proportional to amount of glycogen present; areas with less glycogen showed higher G-6 Pase activity than areas with more glycogen. ALPase activity was increased in livers of animals with severe infections; it was found (only) in neutrophils which were increased under conditions of amebic infection. Acid Pase activity was higher in non-infected animals than in those with very severe infections, and less diffuse, but was highest in animals with lower grade amebic infections. ATPase and DPNH activity was higher in the more severely infected animals than in the controls. Only in one animal, which was moribund when sacrificed, was the activity of liver enzymes generally depressed.

Significance to Biomedical Research and the Program of the Institute:

Amebiasis with its many manifestations and sequelae continues to be one of the more important and most prevalent infectious diseases of the world. It is a leading cause of death in many regions including our neighboring Mexico. One of the major sequelae reported for amebic infection is diffuse, non-invasive amebic hepatitis (especially in India and Mexico) a subject about which there is much difference of scientific opinion. The present studies are an attempt to gain some knowledge of the alleged syndrome under experimental conditions.

Proposed Course of Project:

To continue along lines indicated herein at such times as fresh samples of other pathogenic strains of amebas and their accompanying flora may be obtained from acute human infections.

Honors and Awards: None

Publications: None

Serial No. NIAID - 111-F
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Histochemistry of the intestine in acute amebic enteritis

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: Akio Takeuchi and H. R. Jervis

Cooperating Units: Department of Experimental Pathology, Walter Reed Army Institute of Research, Washington, D.C.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 2/12 | | | 2/12 |
| Professional: | 1/12 | | | 1/12 |
| Other: | 1/12 | | | 1/12 |

Project Description:

Objectives:

To ascertain the effects of amebic infection of the intestine on intestinal enzyme activity under experimental conditions.

Methods Employed:

Germfree guinea pigs are inoculated intracecally with Entamoeba histolytica and the accompanying flora from acute cases of human amebic enteritis. Histochemical determinations are made of the enteric tissues at various stages of the infections.

Major Findings:

With techniques used to stain mucosubstances (alcian blue-PAS high iron-diamine-alcian blue) the brush borders show sulfomucins only. Columnar cells on the surface contain in the apical cytoplasm a number of fine granules of mostly neutral mucosubstances to which may be focally added traces of sulfomucins. Goblet cells, which are rare on the luminal surface but more numerous at the bottoms of the crypts, contain neutral and sulfomucins in various proportions with only occasional traces of sialomucins. The severe discharge of goblet cells

is restricted to parasitized areas and the mucosubstances appear to be concentrated in the supranuclear region of these cells (Golgi region) and these show a stronger reaction for sulfomucins than is found in the controls. Similar concentrations of mucosubstances are found also in columnar cells of the crypts. PAS stained the goblet cells at the bottom as well as those on the lateral wall of the crypts. Large macrophages stained weakly or moderately with PAS. There were small strongly PAS+ cells among the inflammatory cells in severely infected animals but their nature was not established. In the lesions, only PAS+ amebas could be recognized with any degree of certainty. Bacteria in the lesions and on the luminal epithelium were also stained by PAS. ORO+ material was found (as usual) in adipose cells, also in certain inflammatory cells in lesions, and occasionally in the apical cytoplasm of surface epithelial cells (absorbed fat?). It appears that the PAS+ cells might be the same as those with ORO+ material. In amebic lesions the activity of all epithelial enzymes was dramatically decreased except ALPase. In the crypts and in the lamina propria elements ALPase activity did not seem to be impaired. Surrounding lesions and tissues were conspicuous because of the presence of large numbers of ALPase+ polymorphonuclear leukocytes. On the other hand, the large ACPase+ and α-glycP-D+ macrophages typical of guinea pig gut were much less numerous in lesions than in normal tissue. Since amebas could not be positively identified, it was not possible to establish if they showed enzyme activity. Bacteria were demonstrable through their oxidative enzyme activity, especially TPNHD. Different kinds of bacteria reacted for different enzymes. The decrease or disappearance of enzymatic activity in lesions parallels findings in many conditions observed in the small bowel. It is of special interest that fat was apparently absorbed in the cecum and this bears further investigation.

Significance to Biomedical Research and the Program of the Institute:

Although amebic enteritis has been described by some of the master pathologists of the world, practically no information is available on the effects of this parasitization on intestinal enzymes. This is an attempt to elucidate this unknown area of a disease that afflicts millions of human beings throughout the world.

Proposed Course of Project:

To obtain additional data along lines indicated herein until a full understanding of the effects of the infection on intestinal enzymes may be reported.

Honors and Awards: None

Publications: None

Serial No. NIAID - 111-G
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The pathogenic determinants in Entamoeba histolytica with special reference to intracellular viruses of non-pathogenic and highly pathogenic strains

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: Louis S. Diamond*
Carl F. T. Mattern**

Cooperating Units: *Laboratory of Parasitic Diseases, NIAID
**Laboratory of Virus Diseases, NIAID

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 2/12 | | | 2/12 |
| Professional: | 1/12 | | | 1/12 |
| Other: | 1/12 | | | 1/12 |

Project Description:

Objectives:

To ascertain why strains of morphologically identical Entamoeba histolytica vary to the extreme in their pathogenic potential and to identify the factor(s) responsible for this phenomenon.

Methods Employed:

Numerous strains of axenically cultivated E. histolytica are inoculated intrahepatically into young adult jirds. The animals are sacrificed at various post-inoculation intervals and evaluated for pathologic changes of amebic origin. The strains of amebas are also studied by electron microscopy for characteristics that might be determinants of their pathogenic index with special reference to their intracellular viruses.

Major Findings:

A number of strains of the ameba comprising formerly pathogenic strains

that became non-pathogenic in axenic culture have failed to produce lesions in the livers of jirds. These strains were all observed by EM to contain a polyhedral virus. Another strain of the ameba that contains a filamentous virus in addition to the polyhedral virus regularly produces invasive amebic lesions in the livers of inoculated jirds.

Significance to Biomedical Research and the Program of the Institute:

For nearly a century many highly skilled investigators throughout the world have sought answers to the question why morphologically identical E. histolytica often fail to evoke symptoms in some of their hosts (human) whereas in others it may kill and does so with great frequency in many areas of the world. Knowledge of factors responsible may enhance the appreciable international efforts directed toward better control and treatment of this disease.

Proposed Course of Project:

Prioral efforts will be to study the viral associates of additional strains of E. histolytica coincidental with evaluating their pathogenic potential in experimental hosts. Efforts are underway to develop virus-free clones of some non-pathogenic strains tested, with a view toward attempting to infect such clones with virus of the two types harvested from the pathogenic strain of above reference. The strain would then again be tested for effects of the virus alone for pathogenicity in jirds as well as preparations of virus accompanying inactivated amebas.

Honors and Awards: None

Publications:

Phillips, B. P., Diamond, L. S., Bartgis, I. L. and Stuppler, S. A.: Results of intracecal inoculation of germfree and conventional guinea pigs and germfree rats with axenically cultivated Entamoeba histolytica. J. Protozool. 19: 498-499, 1972.

Phillips, B. P.: Concurrent irreversible loss of infectivity-pathogenicity and encystment potential in a strain of Entamoeba histolytica after prolonged maintenance in axenic culture in vitro. Exp. Parasitol. (in press).

Serial No. NIAID - 111-H
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Primary amebic meningoencephalitis due to free-living amebas of the genus Naegleria

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: None

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 5/12 | | | 5/12 |
| Professional: | 4/12 | | | 4/12 |
| Other: | 1/12 | | | 1/12 |

Project Description:

Objectives:

To assess the inherent pathogenicity of this free-living species of ameba in an animal free of other microorganisms and to evaluate several potential methods of entry of the ameba into the host.

Methods Employed:

Free-living amebas of the genus Naegleria, derived from a fatal case of amebic meningoencephalitis in a 27-year old male in Richmond, Virginia, are inoculated intranasally, intraorally, into the conjunctival sac near the inner canthus of the eyes, and into induced skin lesions in germfree guinea pigs. Animals that die or are sacrificed are examined for lesions or tissue changes in the brain, lungs, liver, spleen and intestines. Germfree isolators are carefully monitored for presence of microorganisms other than the ameba.

Major Findings:

Germfree guinea pigs inoculated intranasally with as few as 18 amebas have succumbed at post-inoculation intervals of from 6 to 11 days with amebic meningoencephalitis. None of the animals inoculated by other routes of adminis-

tration have developed symptoms and all appeared normal when sacrificed up to the 84th post-inoculation day. However, the germfree animals (which are a superb culture medium) have uncovered an unidentifiable diphtheroid that grows as an intracellular symbiont in this strain of Naegleria; this symbiont was not detectable by previously employed bacteriological procedures. Drug sensitivity studies of the diphtheroid have indicated its sensitivity to ampicillin and lincomycin and after six months treatment with a combination of the 2 antibiotics it is hoped that the ameba have been freed of this symbiont.

Significance to Biomedical Research and the Program of the Institute:

There have been at least 57 fatal cases of primary amebic meningoencephalitis, attributable to this free-living ameba, reported from countries as remotely apart as U.S.A. (Virginia and Florida), Czechoslovakia, Australia and England. All cases were rapidly fatal, unresponsive to drugs and all were believed to have been acquired while swimming. The organism has again been shown experimentally in the present studies to be capable of penetrating the nasal mucosa and reaching the brain. This appears almost surely to be its method of entering the host. This ameba is a very ubiquitous organism that inhabits fresh water ponds, lakes, streams and even moist soil throughout the world. It is insensitive to 10 p.p.m. chlorine and has been isolated from chlorine-treated swimming pools and even from treated tap-water. It remains unknown why this previously harmless ameba has within the past few years become a dreaded pathogen (some strains). There is speculation that this may be a new strain, or that it may be a case of evolutionary transformation in which case it presents a potential public health problem of magnanimous magnitude. It is imperative that as much information as possible be obtained as rapidly as possible regarding this ameba and the invariably fatal disease it produces. It is not inconceivable that epidemics of appreciable proportions could erupt in any country of the world at any time.

Proposed Course of Project:

Amebas treated with antibiotic and hopingly freed of the intracellular symbiont will be tested in germfree guinea pigs. This symbiont may be used to infect non-pathogenic strains of the ameba for possible influence. Other strains will be tested as described herein. Cultures will be studied by EM for possible intracellular viruses. Chemotherapeutic agents will be screened for activity in vitro against the Naegleria and the germfree guinea pig will be evaluated as a model for drug screening procedures with subsequent testing of chemotherapeutic agents against the disease in these hosts. There is an urgent need for more of any kind of accurate information about this ameba, and there is a most urgent need for an effective chemotherapeutic agent. There have been no survivors of these infections.

Honors and Awards: None

Publications: None

Serial No. NIAID - 113
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The genetic, cellular and regulatory factors in autoallergic disease

Previous Serial Number: Same

Principal Investigator: Philip R. B. McMaster

Other Investigators: Richard Asofsky, LMI, NIAID
Michael Kyriakos*
Gordon Sharp**
Vernon Wong***

Cooperating Units: *Department of Pathology, Barnes Hospital, St. Louis, Missouri
**Department of Medicine, University of Columbia Medical Center, Columbia, Missouri
***Department of Ophthalmology, Georgetown University, Washington, D.C.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 24/12 | | | 24/12 |
| Professional: | 10/12 | | | 10/12 |
| Other: | 14/12 | | | 14/12 |

Project Description:

Project Subtitle I: Cell populations involved in the production of autoimmune disease

Objectives:

To determine if one or more specific subpopulations of leukocytes are responsible for experimental autoimmune disease.

Methods Employed:

Guinea pigs were immunized with guinea pig thyroid extract emulsified in Freund's complete adjuvant. Six to eight days later the lymph node cells from the draining lymph node were harvested. These were then fractionated on a

discontinuous albumin gradient, and the fractions injected into recipient guinea pigs that were histocompatible with the cells. Twelve days later the recipient animals' thyroid glands were removed, and fixed in formalin, sectioned and stained with hematoxylin and eosin for histologic examination in order to determine the frequency and severity of thyroiditis.

Major Findings:

In the preliminary work done to date the light density cells have been the ones that produced thyroiditis in the recipients.

Significance to Biomedical Research and the Program of the Institute:

An identification of the cells that produce autoimmune diseases would enable further analysis of their role in the production of disease.

Proposed Course of Project:

To repeat the work to determine the minimal number of cells required and then to examine their possible cooperative role with other populations.

Project Subtitle II: Effect of irradiation of leukocytes upon the transfer of allergic thyroiditis

Objectives:

To determine which cell properties, such as multiplication, viability, enzyme function and surface properties, must exist for the cells to transfer autoallergic disease, and to measure the effect of adding antigen and antibody to the transferred cells.

Methods Employed:

In addition to those transfer studies previously described, the leukocytes obtained from immunized animals were either irradiated with 4,000 R *in vitro* before being injected into recipient animals or suspended in a large amount of antigen. Selected recipients were given guinea pig anti-thyroid antibody.

Major Findings:

The ability of leukocytes from immunized donors to transfer thyroiditis withstands heavy irradiation - 4,000 R. It is of interest that that dose should suffice to prevent the cells from multiplying in the recipient. The incidence and severity of thyroiditis in the recipients was not markedly affected by adding large amounts of antigen to the cells or by giving the recipients guinea pig antibody repeatedly.

Significance to Biomedical Research and the Program of the Institute:

These results suggest that the irradiated leukocytes act upon the recipients' leukocytes, which in turn produce the disease. This finding will enable investigators to study the disease-producing mechanism of a cell population in a much

more controlled manner than heretofore and may therefore serve to help analyse the role of leukocytes in the formation of autoimmune disease.

Proposed Course of Project:

To label the cells, irradiate them, and then study their distribution in the recipients, to alter the cell surface properties in order to determine which are requisites for successful transfers.

Project Subtitle III: Allergic ocular inflammation

Objectives:

To employ the model system, experimental allergic uveitis, to clarify genetic and pathogenic aspects of autoimmune disease mechanisms.

Methods Employed:

Selected strains of guinea pigs were immunized with guinea pig retina, or with retina and bovine gamma globulin, all emulsified in Freund's complete adjuvant. They were reimmunized 4 weeks later and thereafter examined for signs of ocular inflammation using the slit lamp and histologic sections. At the end of the study they were skin tested with retinal extract, and then bled to carry out Ouchterlony tests for antibody.

Major Findings:

Guinea pigs immunized with retina and a competing antigen, Bgg, and then reimmunized with retina developed delayed hypersensitivity to retina but did not develop uveitis. By contrast, those immunized with retina alone and reimmunized with retina did develop delayed sensitivity to retina and uveitis as well.

Attempts to produce allergic uveitis in different strains of guinea pigs showed that the NIH strain and Hartley strain develop severe disease, whereas Strain 13 guinea pigs produce minimal to mild disease, and Strain 2 animals do not develop it at all. All strains make delayed sensitivity and precipitating antibody to retina.

Significance to Biomedical Research and the Program of the Institute:

These studies show that antigenic competition can prevent the onset of allergic uveitis without preventing the development of delayed sensitivity to at least one of the retinal antigens. This indicates that either delayed sensitivity alone is not a sufficient cause of allergic uveitis, or that if it is a cause of uveitis, then it is only effective if directed to certain of the antigenic determinants of the uvea.

In addition, the work shows that there are strain differences in the proclivity of guinea pigs to produce uveitis, which suggest that genetic differences may be operative.

Future Course of Project:

The resistance of Strain 2 guinea pigs to develop allergic uveitis should be tested using Strain 2 antigen rather than antigen derived from other strains. If the Strain 2 proves resistant, the genetics could be examined to see if predications could be made concerning the likelyhood that humans with ocular injury or infection would develop sympathetic ophthalmia.

In addition, the antigenic competition studies may be repeated with purified retinal antigens to determine if the results depend upon any particular one of the retinal antigens.

Honors and Awards: Chairman of two sessions at 1973 FASEB Meetings on Autoimmunity for the Immunology Society and Autoimmunity for the Experimental Pathology Society

Publications:

Aronson, S. B. and McMaster, P. R. B.: Mechanisms of the host response in the eye. VI. Immune and toxic stimulation of inflammation in the germfree guinea pig. AMA Arch. Ophthal. 58: 533-539, 1972.

McMaster, P. R. B., Powers, K. G., Finerty, J. F. and Lunde, M. N.: The effect of two chlorinated lincomycin analogues against acute toxoplasmosis in mice. J. Trop. Med. Hyg. 22: 14-17, 1973.

Serial No. NIAID - 113-E
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunologic tolerance

Previous Serial Number: Same

Principal Investigator: Philip R. B. McMaster

Other Investigators: Richard Asofsky, LMI, NIAID
William Paul*
Gerald Schiffmann**
John F. Finerty, LMI, NIAID
Cornelius Glaudemans***

Cooperating Units: *Laboratory of Immunology, NIAID, NIH
**Down State Medical School, Brooklyn, New York
***Laboratory of Chemistry, NIAMDD, NIH

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 6/12 | | | 6/12 |
| Professional: | 2/12 | | | 2/12 |
| Other: | 4/12 | | | 4/12 |

Project Description:

Project Subtitle I: The relation of molecular structure to the induction of tolerance

Objectives:

To evaluate the relation of molecular structure and the consequent physiochemical characteristics upon the induction and maintenance of tolerance.

Methods Employed:

Type III pneumococcal polysaccharide was coupled to proteins through nitrobenzoyl derivatives or by the use of cyanuric chloride, giving a firmly linked chemically stable complex. The former was soluble, whereas the latter could be divided into soluble and insoluble fractions. The antibody is evaluated by agglutinin tests and by radioimmunoassay.

Major Findings:

The soluble complex by itself or with adjuvant did not yield detectable antibody in mice. In some rats and all guinea pigs this complex gave high titers when inoculated with adjuvant.

Although the guinea pigs did not make measurable antibody in response to an injection of Type III polysaccharide, inoculating them with large amounts of Type III polysaccharide did alter their response to a complex of that polysaccharide and protein. One mg of polysaccharide completely suppressed the animals' response to immunization, whereas 100 γ did not do so. This appeared to be the case regardless of the method used to detect antibody.

In mice the effect of presenting the tolerogen as an insoluble complex with protein was studied. This type of insoluble complex led to tolerance or immunity in the same dose ranges as did the free soluble material. Similarly, polysaccharide cross linked to itself and insolubilized thereby, also induced tolerance when 1 mg of such material was inserted into the mouse.

Significance to Biomedical Research and the Program of the Institute:

The work with guinea pigs shows that tolerance to the polysaccharide can be established in an animal that does not make antibody after an injection of the native material. The process therefore does not seem to operate by killing cells that start to make antibody but by some other means. The concentration required is in the range of 2.5 γ per gm of animal, which is remarkably close to the concentration required in the mouse and monkey, suggesting that this type of tolerance may somehow be related to either the concentration in the cellular milieu or the amount available per cell.

The work with insoluble products suggest that the immediate form of presentation of the material to the cells may not be important, either because the antigen is eventually solubilized or else because truly insoluble materials are as effective as soluble ones as toleragins.

Proposed Course of Project:

The metabolic requirements for the induction of tolerance will be evaluated in these species of animals. The role of the physiochemical form of the antigen will be pursued to determine if the antigen need ever be soluble to produce tolerance, and to evaluate the effect of the number of determinant groups versus the number of molecules in the process.

Project Subtitle II: Tolerance induction mechanisms to dinitrophenol

Objectives:

To study the mechanisms of tolerance to dinitrophenol, a molecule that is considered to be a T-cell dependent haptene when on a T-cell dependent carrier; and a B-cell dependent haptene, when on a B-cell dependent carrier.

Methods Employed:

Dinitrophenol lysine can be coupled to a polymer of sucrose called Ficoll. When given in sufficient quantity to mice they cannot be immunized with DNP-KLH in adjuvant. If already immunized with DNP-KLH, the DNP Ficoll preparation turns off the response.

Significance to Biomedical Research and the Program of the Institute:

This model system, by virtue of its extreme protracted efficacy, may be very useful for the study of the induction of tolerance.

Proposed Course of Project:

To determine its effect on T- and B-cells, and to evaluate the role of the substitution and molecular size upon the induction of tolerance.

Project Subtitle III: Regulatory mechanisms in DNP antibody production and tolerance in myeloma cells

Objectives:

To determine what regulatory mechanisms may exist in myeloma cells making antibody to DNP.

Methods Employed:

MOPC 315 myeloma cells making antibody to DNP are injected into mice along with enough DNP Ficoll to make the mouse totally tolerant. Serum antibody is measured at intervals thereafter by radioimmunoassay.

Major Findings:

Serum antibody appeared in the mice given tolerizing doses of DNP Ficoll and MOPC tumor cells slightly earlier than in those given just tumor cells. Thus, the tumor cell antibody formation could not be turned off as could ordinary antibody formation.

Significance to Biomedical Research and the Program of the Institute:

This inability of the myeloma cells to become tolerant suggests that they may have lost their suppressor mechanism. A comparison of those cells with normal antibody forming cells may help identify the cell constituents responsible for tolerance. Also, this shows that large quantities of the antigen do not create tolerance by the Treadmill hypothesis mechanism, i.e. by binding the antibody digesting antibody selectively, and liberating the antigen to begin the process again. Were that the case, serum antibody would not have appeared.

Proposed Course of Project:

To be repeated, and analysed with other tumors. Attempts to supply suppressor mechanism molecules to the tumor cells will be made.

Honors and Awards: None

Publications:

Friedlander, M. H., Baer, H. and McMaster, P. R. B.: Contact sensitivity and immunologic tolerance in germfree guinea pigs. Proc. Soc. Exp. Biol. Med. 141: 522-526, 1972.

McMaster, P. R. B., Powers, K. G., Finerty, J. F. and Schiffman, G.: Tolerance to Type III pneumococcal polysaccharide in monkeys. Immunol. Commun. (in press).

Serial No. NIAID - 116
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Control of immunoglobulin synthesis

Previous Serial Number: Same

Principal Investigator: Richard Asofsky

Other Investigators: Martha B. Hylton, LMI, NIAID
Vilho J. Pasanen, LMI, NIAID
Rose Lieberman, Laboratory of Immunology, NIAID
Elizabeth B. Mushinski*
Michael Potter*
Alexander R. Lawton, III**
Max D. Cooper**
Carl Pierce***
Joseph M. Davie****

Cooperating Units: *Laboratory of Cell Biology, NCI
**University of Alabama School of Medicine, Birmingham, Alabama
***Department of Pathology, Harvard Medical School, Cambridge, Massachusetts
****Washington University School of Medicine, St. Louis, Missouri

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 59/12 | | | 59/12 |
| Professional: | 22/12 | | | 22/12 |
| Other: | 37/12 | | | 37/12 |

Project Description:

Objectives:

To elucidate the genetic, developmental and environmental factors which govern expression of immunoglobulin and antibody synthesis with respect to levels in serum and relative rates of synthesis of various immunoglobulin classes.

Methods Employed:

Immunoglobulin levels are measured by radial immunodiffusion using agarose plates impregnated with monospecific antisera. Biosynthesis is studied by incubating tissues in vitro in a medium containing C-14 labeled amino acids. Media are then tested for newly synthesized proteins by means of radioimmunoelectrophoresis. Antibodies are measured by passive hemagglutination, by use of specific immunoadsorbents, by a complement-dependent bactericidal assay and by determining class-specific antibody-forming cells by localized hemolysis-in-agarose gel. Plasma cell tumors are induced by inoculation intraperitoneally of light mineral oil or pristane. Immunoglobulin synthesis is suppressed in vivo by inoculation from birth of appropriate antibodies to mouse immunoglobulins. Synthesis of anti-erythrocyte antibodies are suppressed in vitro by adding appropriate antisera to mouse immunoglobulins to mouse spleen cells in a Mishell-Dutton System. Surface immunoglobulin staining of lymphocytes is accomplished by mixing fluorescein conjugated antibodies to immunoglobulins with cells. Germfree mice are employed when a controlled environment is required.

Major Findings:

Treatment of germfree mice with anti-IgM from birth results in the suppression of all components of the "B" cell line; immunoglobulins are absent from serum, immunoglobulin-bearing lymphocytes are absent from the spleen, plasma cells are virtually absent from all tissues and anatomic regions occupied primarily by "B" lymphocytes, such as germinal centers, and Peyer's patches are very reduced in size. Mice treated in this way fail to make antibody responses, and their cells fail to adoptively transfer the capacity to make antibody responses. These cells are normal, however, with respect to their capacity to produce a GVH reaction, a "T" lymphocyte function. Similar treatment with antibodies to other immunoglobulins fails to produce the above-listed lesions. However, initial treatment with anti-IgM, which is ineffective by itself, followed by treatment with anti-IgG does produce a defect in IgG synthesis.

Anti-IgM suppresses synthesis of all classes of antibody to sheep erythrocytes by unprimed cells in vitro. Anti-IgG and anti-IgA produce suppression of those specific classes only. Anti-IgM suppresses synthesis only of IgM synthesis by hyperimmune cells. The same degree of suppression by anti-IgG and anti-IgA is produced in primed as in unprimed cells.

AbL mice, bred for low responses to erythrocyte antigens, have very low immunoglobulin levels. The low immunoglobulin levels are a heritable trait which can be dissociated from the immune response to sheep erythrocytes (used for selection), and pneumococcal polysaccharide Type III.

Lymphocytes bearing immunoglobulins on their membranes develop almost identically in spleens with age in germfree as in conventionally-reared mice. The predominant immunoglobulin class is IgM. There is about a 400-fold increase in IgM-bearing lymphocytes from birth to one week of age, and only a three-fold further increase from one to eight weeks of age. IgM-bearing cells are almost absent at birth.

Sera from unimmunized germfree mice contain only $7S\gamma_1$ and IgM. Immunization with ferritin or other proteins results in a transient increase in IgM and a

sustained increase in $7S\gamma_1$ . A second injection of the same (but not another) antigen results in a sustained increase in IgM levels, further increases in $7S\gamma_1$, and the first appearance of $7S\gamma_2$. Specific antibody accounts for less than 10% of the IgM, less than 20% of the $7S\gamma_1$, and less than 40% of the $7S\gamma_2$. Increasing the interval between the first and second injection of antigen results in increased antibody titers, with no increase in the fraction of each immunoglobulin that can be accounted for as specific antibody.

Intraperitoneal inoculation of an endotoxin results in a sustained increase in IgM with doses of antigen between $5 \times 10^{-4}$ µg and 50 µg. IgM and bactericidal levels are well correlated, but less than 10% of the IgM is specific antibody. Inoculation of a "native protoplasmic polysaccharide" from E. coli, a material with molecular weight about 110,000, results in increases in IgM levels comparable to those seen with endotoxins.

Germfree BALB/c mice inoculated with light mineral oil or pristane develop very few plasma cell tumors in contrast to the high incidence obtained in conventional animals. Germfree BALB/c mice monocontaminated with bacteria or fed dead bacteria, then inoculated with mineral oil or pristane, develop plasma cell tumors in much higher incidence than do uncontaminated mice, but in lower incidence and much later than conventional mice. All of the tumors produced by monoinfected germfree mice that have been examined thus far produced IgA myeloma proteins. Mineral oil or pristane granulomas synthesized small amounts of all classes of immunoglobulins within one week of inoculation. There is an incidence of reticulum cell sarcoma of about 15% in all aged (> 18 months) germfree BALB/c mice.

Certain of the above lymphoid tumors are transplantable. At least one, and probably several such tumors, if given to syngeneic mice at the time of immunization, amplify the immune response to the immunizing antigen.

Significance to Biomedical Research and the Program of the Institute:

Mice treated from birth with anti-immunoglobulin can show complete or selective hypogammaglobulineamia. The conditions produced resemble certain forms of human hypogammaglobulineamia and may serve as models, as may AbL mice, for those disorders. Furthermore, the role of antibody in resistance to infection can be evaluated best in such animals.

Nonspecific stimulation of antibody formation by syngeneic lymphoid tumors suggests that the neoplastic cells may elaborate a product which enhances the differentiation of antibody-forming cells.

The parallel development of "B" lymphocytes in germfree and conventional mice suggests that this step in differentiation does not require antigen.

Proposed Course of Project:

Subpopulations of AbL mice with respect to capacity to respond to a variety of antigens will be defined and bred. Mice suppressed with anti-IgM from birth will be used in studies of resistance to viruses, bacteria, parasites and tumor cells. Transplantable lymphoid tumors will be examined further for capacity to

mediate normal physiologic function.

Honors and Awards: None

Publications:

Pierce, C. W., Asofsky, R. and Solliday, S.: Immunoglobulin receptors on B lymphocytes: Shifts in immunoglobulin class during immune responses. Fed. Proc. 32: 41-43, 1973.

Anderson, L. G., Cummings, N. A., Asofsky, R., Hylton, M. B., Tarpley, T. M., Tomasi, T. B., Wolf, R. O., Schall, G. L. and Talal, N.: Salivary gland immunoglobulin and rheumatoid factor synthesis in Sjögren's syndrome. Natural history and response to treatment. Am. J. Med. 53: 456-463, 1972.

Serial No. NIAID - 116-J
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The suppressive effects of heterologous antithymocyte sera on the homograft reactions in mice

Previous Serial Number: Same

Principal Investigator: Herbert C. Morse, III

Other Investigators: Richard Asofsky, LMI, NIAID
Robert E. Tigelaar*
Marek Cwynarski**

Cooperating Units: *Working assignment, University College, London, England
**Immunology Branch, NCI

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 36/12 | | | 36/12 |
| Professional: | 12/12 | | | 12/12 |
| Other: | 24/12 | | | 24/12 |

Project Description:

Objectives:

To investigate the mechanism of action of antithymocyte sera in suppressing graft-versus-host reactions and allograft rejection.

Methods Employed:

Antithymocyte sera (ATS) were prepared by immunization of rabbits with two intravenous injections of BALB/c thymus cells. Graft-versus-host assays wereperformed by inoculating spleen cells from mice into newborn allogeneic recipients, and measuring the resulting splenomegaly 9 days later. The activity of cells from animals receiving a single dose of ATS was measured with a GVH assay 2, 11 and 30 days after treatment. In addition, spleen cells from ATS-treated mice were labelled with chromium-51 and injected into lethally irradiated syngeneic recipients. Twenty-four hours later the mice were sacrificed and tissues removed for counting in an assay for the effects of ATS on cells migrating to various organs--spleen, peripheral lymph node (PLN), mesenteric

lymph node (MLN), liver, lung and thymus.

Major Findings:

Pretreatment of donors with 0.1 ml of ATS produced an 83% reduction in the GVH reactivity of spleen cells with a gradual recovery to 64% of normal reactivity at 30 days after ATS administration. By comparison, ATS effected a 60% reduction in the capacity of cells from donor spleens to migrate to lymph nodes, either PLN or MLN, at 2 days after ATS but there was a rapid and complete return of this function to normal levels by day 30. Studies of the migratory capacity of cells from spleens of "nude" athymic mice in combination with evaluation of the capacity of anti-theta antiserum to release counts from cells migrating to LN demonstrated that the predominant cell type migrating to LN was thymus-derived. (See also NIAID - 118 for ATS effects on direct and synergizing GVH activity in LN.)

Significance to Biomedical Research and the Program of the Institute:

ATS is a potent and useful immunosuppressant which has found important clinical use. These studies have yielded further supportive evidence for the role of more than one T-cell population participating in the GVH response and indicate that the T-cell population which migrates to LN from spleen cannot be equated with that responsible for GVH activity in spleen. At least one cell component responsible for splenic GVH reactivity is much more ATS-sensitive than those cells which have LN-seeking capacity.

Proposed Course of Project:

The capacity to differentially effect subpopulations of thymus-derived cells with ATS treatment will be examined further to characterize the immune potentials of T-cells from distinct lymphoid compartments in terms of their GVH potential--both direct and synergizing--as well as their ability to participate in humoral responses.

Honors and Awards: None

Publications:

Cantor, H. and Asofsky, R.: Paradoxical effects of antithymocyte serum on the thymus. Nature 243: 39-41, 1973.

Serial No. NIAID - 117
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The immunologic response of conventional and germfree mice to blood-induced infections of Plasmodium berghei

Previous Serial Number: Same

Principal Investigator: John F. Finerty

Other Investigators: Charles B. Evans, LMI, NIAID

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 30/12 | | | 30/12 |
| Professional: | 6/12 | | | 6/12 |
| Other: | 24/12 | | | 24/12 |

Project Description:

Objectives:

To immunize mice against Plasmodium berghei. To determine the dose of malarial antigen that will induce protective immunity in mice. To determine the time interval required for protective immunity to develop between antigen injection and parasite challenge.

Methods Employed:

P. berghei parasites were removed from their erythrocytes and inactivated with formaldehyde treatment or heat. The treated parasites were injected intraperitoneally (IP) or subcutaneously (SC), without adjuvants, in multiple doses. Serum antibody was measured by the indirect fluorescent antibody technique or by parasite agglutination. Non-immunized and immunized mice were challenged at various intervals with live P. berghei.

Major Findings:

Antigenic preparations from strains of Plasmodium berghei revealed different serologic properties. Agglutination of P. berghei, NYU-2, antigen did not occur

in sera prepared from normal infected or immune sera, despite the fact that immune animals were resistant to the NYU-2 strain. However, antigen smears prepared from all strains of P. berghei gave positive results, i.e. fluorescence with sera from infected or immune animals.

A comparison of the immunizing properties of P. berghei revealed that NYU-2 antigen conferred the least degree of resistance in mice. Both IP and SC routes of immunization were used, with the latter slightly more effective.

Significance to Biomedical Research and the Program of the Institute:

The ability to induce protective immunity against P. berghei without the use of adjuvants indicates that the antigen(s) involved are not destroyed during inactivation. The data suggest that virulence and immunogenicity show an inverse relationship. These experiments indicate (1) that the best vaccines may be obtained from strains of P. berghei that are not virulent, and (2) that induction of antibody formation is unnecessary in conferring protection.

Proposed Course of Project:

Future studies will involve the identification and isolation of functional antigen(s) responsible for malarial immunization.

Honors and Awards:

Invited to give a seminar at the New York University School of Medicine, School of Tropical Medicine, December, 1972. Title of seminar:

"Immunologic Parameters in Malaria and Trypanosome Infections"

Publications:

Finerty, J. F., Tobie, J. E. and Evans, C. B.: Antibody and immunoglobulin synthesis in germfree and conventional mice infected with Plasmodium berghei. Am. J. Trop. Med. Hyg. 21: 499-505, 1972.

Hyde, C. L., Finerty, J. F. and Evans, C. B.: Antibody and immunoglobulin synthesis in germfree and conventional mice infected with Eperythrozoon coccoides. Am. J. Trop. Med. Hyg. 21: 506-511, 1972.

Ziegler, J. L., Bluming, A. Z., Morrow, R. H., Cohen, M. H., Fife, E. H., Finerty, J. F. and Woods, R.: Burkitt's lymphoma and malaria. Trans. Roy. Soc. Trop. Med. Hyg. 66: 285, 1972.

Finerty, J. F., Hyde, C. L. and Evans, C. B.: Plasmodium berghei and Eperythrozoon coccoides: Antibody and immunoglobulin synthesis in germfree and conventional mice simultaneously infected. Exp. Parasitol. (in press).

McMaster, et al. (cf NIAID - 113).

Serial No. NIAID - 117-C
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Separation of different erythrocytic stages of Plasmodium berghei and P. knowlesi

Previous Serial Number: Same

Principal Investigator: John F. Finerty

Other Investigators: Charles B. Evans, LMI, NIAID

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 8/12 | | | 8/12 |
| Professional: | 4/12 | | | 4/12 |
| Other: | 4/12 | | | 4/12 |

Project Description:

Objectives:

To isolate the various erythrocytic stages of Plasmodium berghei and P. knowlesi. To concentrate, isolate and utilize these stages in studies concerning the immune response to malaria.

Methods Employed:

Whole blood parasitized with P. berghei was obtained from BALB/c mice. Parasites were separated on discontinuous density gradients and then freed from their erythrocytes by ammonium chloride lysis. The parasites were stained with Giemsa solution for microscopic examination. Parasites were used for the detection of rosette-forming cells (RFC) or for agglutination. RFC were formed on mixing of appropriate leukocytes with parasites; a leukocyte with two or more adherent parasites was considered an RFC.

Major Findings:

RFC were detected with the peripheral white cells of mice infected with P. berghei. Only parasites freed from their erythrocytes formed rosettes;

parasitized erythrocytes did not. Seven days after inoculation with live parasitized erythrocytes, 25% of the peripheral leukocytes were RFC. Rosettes were also observed with peripheral lymphocytes obtained from mice resistant to P. berghei. Approximately 4% of these cells were RFC. Only trophozoites, ring forms and merozoites were observed to form rosettes; schizonts did not adhere to white cells.

Parasitized erythrocytes and schizonts inactivated with formaldehyde gave different agglutination results with sera from infected animals. Schizonts were agglutinated within 21 days after parasite inoculation whereas parasitized erythrocytes did not agglutinate within this same time interval. Antigenicity of these preparations was not lost upon storage at $10^{o}$C for as long as 6 months.

Significance to Biomedical Research and the Program of the Institute:

Inactivation of malarial schizonts with formalin and storage at $4^{o}$C without loss of antigenicity adds a new dimension to the immunologic study of host immune response to malaria. Schizont antigens can be used to assay agglutinins present in the sera of infected individuals, and correlate these results with different states of immunity. In addition, formalinized schizonts used in the schizont-agglutination assay should be readily adaptable to field studies.

The rosette formation by freed malarial parasites indicates further that the parasites retain their antigenic properties after being released from their erythrocytes. The results further suggest that RFC may detect malarial antigens or antibody not detected by other serologic procedures. In addition, the determination of the type of cells involved in functional immunity to malaria will be facilitated by rosette technique.

Proposed Course of Project:

Parasites treated with formalin will be used to study the kinetics of the humoral antibody response to malarial organisms. Attention will focus on animals that recover from infection in an attempt to elucidate the nature of the antibody/antigen which confers resistance to the host. Absorption studies will be used to determine whether the specificity of antibody agglutinating schizonts and that agglutinating parasitized erythrocytes is different.

Future studies with the rosette technique will involve the types of leukocytes involved and the immunoglobulins on their surfaces. Additional studies will determine whether RFC are thymus or bone-marrow derived.

Honors and Awards: None

Publications: None

Serial No. NIAID - 117-E
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The immunologic response of animals immunized with trypanosomes

Previous Serial Number: Same

Principal Investigator: John F. Finerty

Other Investigators: Philip R. B. McMaster and Charles B. Evans, LMI, NIAID

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 6/12 | | | 6/12 |
| Professional: | 2/12 | | | 2/12 |
| Other: | 4/12 | | | 4/12 |

Project Description:

Objectives:

To determine the immunoglobulins produced and classes of antibody synthesized in animals immunized against Trypanosoma rhodesiense. To determine the efficacy of the immunization procedures by challenge with live T. rhodesiense.

Methods Employed:

Rabbits were injected with either live T. rhodesiense or formalin-killed organisms. Animals initially infected were subsequently immunized with trypanosomal antigens. Other animals that had initially been immunized were challenged with live organisms. Serum antibody was measured by trypanosome agglutination and indirect fluorescent antibody techniques (IFA). All animals were skin tested at the end of the studies.

Major Findings:

Rabbits infected with live T. rhodesiense succumb to the infection in 6-9 weeks after inoculation. However, infected animals that were immunized with trypanosomal antigen during the course of their disease usually recovered. Immunized animals that showed no detectable serum antibody by agglutination or IFA were resistant upon challenge with live trypanosomes.

Normal infected and resistant animals were skin tested with a soluble antigenic preparation. Normal and infected animals had negative skin tests, but all resistant animals demonstrated skin lesions that reached maximum diameters within 18-24 hours. The largest reactions were found in animals that had initially been infected and then immunized. Immunized, resistant rabbits had no detectable serum antibody.

Significance to Biomedical Research and the Program of the Institute:

This work indicates that an immunotherapeutic approach to the treatment of parasitic infections is feasible. The lack of detectable serum antibody in protected animals supports the conclusion that humoral antibody has little effect in conferring protection against trypanosomiasis. This argument is strengthened by the positive skin tests observed in resistant animals with no detectable serum antibody. These data suggest that immunity to trypanosomes is a function of cell-mediated immunity.

Proposed Course of Project:

Future studies will focus on the antigen(s) involved in the induction of a protective immune response. The roles of humoral and cellular immunity will be studied further in relation to infection and active immunization. The specificity of protection and the delayed hypersensitivity reactions accompanying it will be determined.

Honors and Awards: None

Publications: None

Serial No. NIAID - 118
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Synergy in the graft-versus-host response

Previous Serial Number: Same

Principal Investigator: Herbert C. Morse, III

Other Investigators: Richard Asofsky, LMI, NIAID
Richard Gershon*
Robert E. Tigelaar**
Michael Harrison***

Cooperating Units: *Department of Pathology, Yale University, New Haven, Connecticut
**Working assignment, University College, London, England
***Laboratory of Immunology, NIAID

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 15/12 | | | 15/12 |
| Professional: | 12/12 | | | 12/12 |
| Other: | 3/12 | | | 3/12 |

Project Description:

Objectives:

To characterize subpopulations of lymphoid cells which interact in the graft-versus-host (GVH) reaction and to elucidate the nature of this interaction.

Methods Employed:

GVH reactions were produced by inoculation of mouse lymphoid cells from tissues such as spleen, lymph nodes (LN), thymus and peripheral blood (PBL) or combinations of such cells into newborn allogeneic or $F_1$ recipients. The degree of GVH activity was assayed by two methods. The Simonsen spleen weight assay, in which the reactivity of the injected lymphoid cells is measured by the degree of spleen enlargement in recipient mice was routinely used. A death assay, in which the cumulative mortality of sublethally irradiated recipients of lymphoid

cells was expressed as a function both of time and of inoculum size was also employed. Some donor mice were pretreated subcutaneously with a single dose of antithymocyte serum and cells from various lymphoid tissues were examined thereafter for their GVH reactivity.

Major Findings:

Studies utilizing the spleen weight assay have indicated that optimal expression of GVH reactions may involve interaction among two or more cell types. These populations have the following characteristics: (1) One population is present in high concentrations in tissues which are rich in recirculating lymphocytes, such as PBL or LN. The second is a population of relatively more sessile cells found in higher concentrations in thymus and spleen. (2) Both populations must have the capacity to recognize foreign antigens in the recipient. (3) Both of the cell types involved are "thymic-dependent." (4) The cell type present in excess in thymocyte suspensions appears to act as a precursor of cells which inflict immunologic injury while the cell present in excess in PBL or LN amplifies the activity of the precursor cells.

A series of experiments were designed to see whether manipulation of normal spleen cells could lead to a more explicit demonstration of the existance of subpopulations of cells important in GVH reactions. Normal parental spleen cells were injected into lethally irradiated syngeneic recipients; 24 hours later the spleens and lymph nodes were removed and these populations tested for their GVH reactivity in a spleen weight assay either directly or combined with each other. Spleen-seeking spleen cells (SSSC) were less reactive than normal spleen cells as indicated both by the greater number of such cells required to produce significant minimum splenomegaly as well as by a significantly flatter dose-response curve, i.e., many more SSSC than normal spleen cells were required to produce a given increment in observed splenomegaly. Lymph node-seeking spleen cells (LSSC) also showed a significantly flatter dose-response curve than either normal lymph node or normal spleen cells. Appropriate combinations of SSSC and LSSC produced splenomegaly greater than expected from summing the reactivities of these populations measured separately. Furthermore, these mixtures restored the steep dose-response curve characteristic of normal spleen cells, the population from which they were derived. As in the previous models, substituting syngeneic $F_1$ cells for either of the passaged parental cell types did not result in synergy. Pretreatment of either of the populations in the mixture of parental cells with anti-theta antiserum and complement resulted in levels of splenomegaly similar to those expected from the untreated population alone. Furthermore, SSSC interacted synergistically with appropriate numbers of normal LN cells as did LSSC when combined with normal thymus cells. These data indicate that within the spleen there exist at least two subpopulations of thymus-derived cells which have different migratory properties in irradiated syngeneic recipients, and that both of these cells are necessary for normal and full expression of the GVH reactivity of a normal peripheral lymphoid tissue.

The effect of delaying the injection of either one of the populations of normal lymphoid cells which when injected simultaneously resulted in synergy was investigated. Injection of LN cells 24 hours after injection of thymocytes resulted in spleen indices similar to those seen when these populations were

injected together. However, injecting thymocytes 24 hours after LN cells resulted in markedly reduced spleen indices--little synergy was seen.

Thymus cell donors were pretreated with 2.5 mg cortisone acetate 48-72 hours prior to sacrifice. Although the specific activity of such cells was markedly increased compared to normal thymocytes, i.e., reactivity similar to that observed with normal spleen cells, nevertheless only approximately 50% of the total reactivity present in a normal thymus could be accounted for by the reactivity of the small number of cells recoverable from the thymus of a cortisone-treated donor. Furthermore, this population of cells were incapable of interacting synergistically with either normal LN cells or with normal thymocytes, indicating that the population of cells in normal thymus which can interact with LN cells or PBL are sensitive to the action of cortisone acetate, and that the cortisone-resistant cells do not contain an excess of cells capable of amplifying the activity of normal thymus cells.

Rather similar results have been obtained following incubation of thymocytes from an appropriate strain of mice with complement and an antiserum directed against an antigen (TL) present on the majority of such thymocytes. The residual population had enhanced specific activity when tested directly, but had lost its capacity to interact synergistically with normal PBL; combination of these cells with normal thymus cells resulted in recipient splenomegaly only slightly greater than expected, i.e., little if any synergy was observed.

Further experiments were designed to determine if these subpopulations of thymus-derived cells represent different stages in the maturation of a single cell line or indicate the presence of two discreet cell populations. PLN from mice treated with ATS 2, 11 and 30 days prior to assay (see NIAID - 116-J) were tested for their direct reactivity in the spleen assay as well as their ability to give synergy with normal thymocytes. Direct reactivity of such cells was markedly suppressed at day 2 (>80%) with minimal recovery by days 11 and 30 with only 40% of normal activity found at these times. However, the ability of these cells to produce synergy with normal thymocytes was supranormal (150% of normal) at day 11. This would be inconsistent with the maturational concept which would suggest that amplifier cells should appear at the expense of precursor cells and is more in keeping with the view that these cell types represent discreetly derived populations with greatly differing ATS sensitivities.

Other investigators have demonstrated that the capacity of parental bone marrow cells to produce GVH mortality in reciprocal $F_1$ recipients is reduced when donor cells are of maternal origin, suggesting the possibility that $F_1$ cells may be rendered less "antigenic" in utero by maternal modulation. This problem was re-evaluated in studies using spleen weight and death assays of the GVH reactivity of paternal and maternal spleen cells (BALB/c or C57Bl/6) in reciprocal $F_1$ recipients (BALB/c x C57Bl/6 or C57Bl/6 x BALB/c). The spleen weight assay demonstrated that the pattern of relative reactivity of parental cells in either recipient was the same with BALB/c spleen cells being more active than those of C57Bl/6 origin. The results of mortality assays are not complete.

Significance to Biomedical Research and the Program of the Institute:

The present studies have shown that different populations of thymus-derived lymphoid cells can interact synergistically in producing GVH reactions quantitated either by mortality or by splenomegaly. This points to a previously unrecognized functional heterogeneity within the peripheral pool of cells involved in cellular immune reactions against foreign tissues. Further delineation of the nature of this interaction and the characterization of the cells involved is obviously necessary for a clearer understanding of this type of immune response. Appropriate adjustments of the cell populations involved in cell-mediated immune responses against tumor antigens might enhance the process of tumor rejection of either syngeneic or autochthonous tumors. Similarly, the elimination of small subpopulations of lymphocytes necessary for full expression of normal GVH reactivity may have significance in dealing with the problem of suppression of homograft rejection, and of the graft-versus-host reactions which complicate bone marrow transplantation.

Proposed Course of Project:

To further define the subpopulations of thymus-derived cells through differential effects of ATS treatment, migration (see NIAID - 116-J) and thymectomy on their physiologic properties. To determine if synergistic protective interaction of these cell types can be demonstrated in studies of T-cell deprived mice infected with Herpes simplex. To test the ability of these cells alone or in combination to reject or prevent growth of syngeneic plasmacytomas in mice.

Honors and Awards: None

Publications:

Tigelaar, R. E. and Asofsky, R.: Synergy among lymphoid cells mediating the graft-versus-host response. V. Derivation by differential migration in lethally irradiated recipients of two interacting subpopulations of thymus-derived cells from normal spleen. J. Exp. Med. 137: 239-253, 1973.

Tigelaar, R. E. and Asofsky, R.: Kinetics of graft-versus-host splenomegaly in neonatal $F_1$ recipients of parental strain mouse lymph node cells and/or thymocytes. Transplantation 14:805-807, 1972.

Tigelaar, R. E. and Asofsky, R.: Graft-versus-host reactivity of mouse thymocytes: Effect of cortisone pretreatment of donors. J. Immunol. 110: 567-574, 1973.

Serial No. NIAID - 119-A
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Quantitative and qualitative studies on the antibody response to pneumococcal polysaccharides at the cellular level

Previous Serial Number: Same

Principal Investigator: Phillip J. Baker

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**Veterinary Resource Branch, Division of Research Services
***Department of Botany and Microbiology, Montana State University, Bozeman, Montana
****University of Alabama School of Medicine, Birmingham, Alabama

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total: | 62/12 | | | 62/12 |
| Professional: | 24/12 | | | 24/12 |
| Other: | 38/12 | | | 38/12 |

Project Description:

Project Subtitle I: Genetic control of the antibody response to Type III pneumococcal polysaccharide (SSS-III)

Objectives:

To characterize those genetic factors that regulate the magnitude of the antibody response to SSS-III and to obtain information concerning their mode of action.

Methods Employed:

The γM antibody response to SSS-III was assessed in $F_1$, $F_2$, and backcross progeny derived from high (BALB/cAnN) and extremely low (CBA/HN) responding parental strains of inbred mice. An adaptation of the technique of localized hemolysis-in-gel was used to detect antibody-producing or plaque-forming cells (PFC) specific for SSS-III, and serum antibody was detected by passive immune hemolysis; sheep erythrocytes, sensitized with SSS-III by the chromium chloride coupling procedure, were used in both of these tests. Serum immunoglobulin levels were measured by radial immunodiffusion using plates containing monospecific antisera. Numbers of bone marrow-derived precursors of antibody-forming cells (B cells) were determined by membrane and tissue fluorescence using monospecific reagents.

Major Findings:

The results of more extensive genetic studies conducted with reciprocal $F_1$, $F_2$, and backcross progeny derived from high and low responding parental strains of mice affirmed that a major component involved in the IgM antibody response to SSS-III is X-linked, i.e., carried on the X chromosome; this component determines responsiveness in an almost "all-or-none" manner. Other factors, presumably autosomal genes, regulate the magnitude of the response elicited by mice possessing the X-linked gene; these appear to influence independently the number of cells participating in the immune response and the amount of antibody made by such cells. High and low responders were found to differ considerably with respect to γM immunoglobulin levels; however, there was no obvious direct relationship between the amount of γM immunoglobulin produced and the ability to respond to SSS-III. Although treatment with endotoxin (LPS) resulted in a substantial increase in the amount of γM immunoglobulin synthesized by both low and high responders, the ability of low responders to make antibody specific for SSS-III and other polysaccharide antigens was still impaired. No striking differences between high and low responders were noted with respect to γ1, γ2 and γA immunoglobulin levels and both groups of mice appear to possess similar numbers of immunoglobulin-bearing B cells.

Significance to Biomedical Research and the Program of the Institute:

Since several immunological deficiency diseases of man have been reported to be X-linked, the above experimental model permits one to identify and characterize some of the genetic factors that may be associated with the development of such diseases. In this context, low γM immunoglobulin levels and an inability to respond to polysaccharide antigens are characteristics associated with Wickett-Aldrich disease.

Proposed Course of Project:

Most of the information obtained thus far indicates that the genetic defects described are confined largely to the B lymphocyte population. The presence of normal numbers of B cells, as well as elevated γM immunoglobulin levels following treatment with LPS, suggests that while low responding mice possess B cells capable of synthesizing immunoglobulin, such cells are unable to make antibody upon stimulation by antigen. Studies are in progress to determine whether this is due primarily to a lack of antigen-specific receptors on B cells, or the inability of such receptors to initiate antibody synthesis

following contact with antigen. Studies are also being conducted to determine whether high and low responding mice differ significantly with respect to functions mediated by thymic-derived cells (T cells); particular attention will be given to functions mediated by regulatory T cells.

Honors and Awards: None

Publications:

Amsbaugh, D. F., Hansen, C. T., Prescott, B., Stashak, P. W., Barthold, D. R., and Baker, P. J.: Genetic control of the antibody response to Type III pneumococcal polysaccharide in mice. I. Evidence that an X-linked gene plays a decisive role in determining responsiveness. J. Exp. Med. 136: 931-949, 1972.

Project Subtitle II: Regulation of the antibody response to SSS-III

Objectives:

To provide more precise information concerning the nature of regulatory cells and the mechanism by which they influence the magnitude of the antibody response to SSS-III.

Methods Employed:

The immunological procedures used to detect serum antibody and PFC specific for SSS-III are given in the preceding section of this report; these procedures were used in conjunction with class-specific antisera for the detection of γG and γA-producing PFC. Horse anti-mouse lymphocyte serum (ALS) was administered at the time of immunization with SSS-III. Unless stated otherwise, the magnitude of the antibody response to SSS-III was assessed at peak, 5 days after immunization.

Major Findings:

The effect of treatment with ALS on the magnitude of the PFC response to SSS-III was assessed in athymic nude, as well as thymus-bearing littermate control, mice. Without ALS treatment, the PFC response was slightly higher in nude, than in control, mice. Treatment with ALS had no effect on the response of nude mice; however, considerable enhancement (about 100-fold) was noted in the case of thymus-bearing controls. Such enhancement was ALS dose-dependent and demonstrable under conditions in which there was substantial or complete inactivation of thymic-derived "helper" cells required for an antibody response to erythrocyte antigens. These findings suggest that amplifier and suppressor cells, which have been reported to regulate the magnitude of the antibody response to SSS-III, represent populations of T cells that are present in normal--but not athymic--mice. The activities of "helper" T cells and regulatory T cells appear to be independent of one another and mediated by separate subpopulations of T cells.

The mode of action of suppressor T cells was established by examining the effects of treatment with ALS on the kinetics for the appearance of SSS-III-specific PFC. Without ALS, the kinetics were essentially biphasic. First,

there was a phase during which PFC appeared at a relatively rapid rate; this was followed by a phase during which the rate of appearance of PFC gradually declined until maximal numbers were attained. In contrast, PFC increased at a uniform exponential rate, throughout the first 5 days of the antibody response in ALS-treated mice. Treatment with Velban, a mitotic inhibitor, completely arrested the development of additional PFC in ALS-treated--but not non-ALS-treated--mice. These findings indicate that regulatory suppressor T cells act primarily by limiting the extent to which antibody-forming B cells proliferate following immunization.

Small--but significant--numbers of γG and γA-producing PFC could be detected in the spleens of mice immunized with SSS-III. Treatment with ALS resulted in a 10 to 20-fold increase in numbers of γM, γG and γA-producing PFC detected. These findings suggest that suppressor T cells, inactivated following treatment with ALS, regulate the amount of all types of antibody produced following immunization with SSS-III.

Significance to Biomedical Research and the Program of the Institute:

The above findings illustrate that suppressor T cells act primarily by limiting the extent to which antibody-producing B cells proliferate following immunization; other investigators have shown that suppressor T cells can influence the antigen-induced proliferative response of other types of T cells. Suppressor T cells, therefore, may provide a homeostatic control mechanism for limiting the clone size of all types of lymphoid cells and thereby play an important role in preventing the development of lymphoproliferative disorders and autoimmune disease.

Proposed Course of Project:

Studies are in progress to determine how suppressor T cells are activated during the course of an immune response, the nature of the product made by such cells, and the role of suppressor T cells in the development of immunological tolerance and competition between antigens. Procedures are being devised to enable one to obtain sufficient numbers of amplifier T cells so that their mode of action can be assessed. A search is being made for suitable strains of inbred mice that can be used in studies on the genetic control of regulatory T cell function.

Honors and Awards: None

Publications:

Baker, P. J., Reed, N. D., Stashak, P. W., Amsbaugh, D. F., and Prescott, B.: Regulation of the antibody response to Type III pneumococcal polysaccharide. I. The nature of regulatory cells. J. Exp. Med. (in press).

Barthold, D. R., Stashak, P. W., Amsbaugh, D. F., Prescott, B., and Baker, P. J.: Strain differences in the ability of antithymocyte serum (ATS) to enhance the antibody response of inbred mice to Type III pneumococcal polysaccharide. Cell. Immunol. 6: 315-323, 1973.

LABORATORY OF PARASITIC DISEASES


| | | |
|---|---|---|
| Summary | ..... | S-1 |
| 120 | Administration from the Office of the Chief ..... | 1 |
| 121-C | The biology and control of the snail intermediate hosts of schistosomes ..... | 4 |
| 121-H | The pathogenesis of schistosome infections in mammalian hosts ..... | 9 |
| 121-I | Serologic studies on filariasis ..... | 13 |
| 121-J | Host-parasite relationships in filariasis ..... | 17 |
| 121-K | Detection and characterization of a circulating schistosome antigen and other immunologic studies in schistosomiasis ..... | 22 |
| 122-G | Studies on Entamoeba histolytica and other parasitic protozoa ..... | 24 |
| 122-J | Biochemical basis for growth and differentiation of parasitic and free-living nematodes ..... | 29 |
| 123-B | Pathological physiology and histochemistry of parasitic diseases ..... | 34 |
| 123-C | Biochemical mechanisms of energy metabolism in mammalian and parasitic organisms ..... | 37 |
| 123-E | Biophysical parasitology ..... | 42 |
| 123-F | Terminal electron transport in parasites ..... | 48 |
| 125-A | Studies on toxoplasmosis ..... | 51 |
| 125-B | In vitro cultivation of Coccidia ..... | 54 |
| 125-I | Electron microscope studies on parasites ..... | 56 |
| 125-K | Amebiasis studies in India ..... | 58 |
| 125-P | Immunological studies on toxoplasmosis and other parasitic diseases ..... | 61 |
| 132 | Administration and planning of section ..... | 65 |
| 132-A | Mechanism of invasion of malarial merozoites ..... | 67 |
| 132-B | Studies on immunity and pathology of malaria and other parasitic diseases ..... | 70 |

| | | |
|---|---|---|
| 132-C | Experimental chemotherapy of malaria and other protozoan diseases | 75 |
| 132-D | Studies on drug resistance in experimental malaria | 78 |
| 132-E | Studies on the labelling of sporozoites of malaria | 81 |
| 132-O | Pathogenesis of disease (Chagas' disease) resulting from infection with Trypanosoma cruzi | 83 |
| 133 | Susceptibility of Anopheline mosquitoes to simian and human malaria infections and their ability to transmit to simian and human hosts | 86 |
| 133-A | Studies of the Exoerythrocytic stages of primate malaria | 90 |
| 133-B | Immuno-ecology of primate malaria | 93 |
| 133-C | Fluorescent antibody studies in simian and human malaria | 96 |
| 133-D | Immunological studies on primate malaria | 98 |
| 133-E | Study of cell-mediated immunity (CMI) in simian malaria | 101 |
| 133-F | The role of malaria in the induction of lymphoma by Epstein-Barr virus (EBV) | 103 |
| 133-G | Studies on gametogenesis and the infectivity of primate malarias to mosquitoes | 105 |
| 133-H | Effects of chemosterilization on the development and transmission of malaria parasites in mosquitoes | 107 |
| 133-I | Comparative histological and histochemical studies on the sporogonic cycle of malaria parasites | 109 |
| 133-J | Gametocytocidal and sporontocidal effects of some commonly used antimalarial drugs on the sporogonic cycle of malaria parasites | 111 |
| 133-K | Studies on the genetics of vector capacity of Anopheline mosquitoes for primate malarias | 114 |
| 133-L | Role of serum complement in primate malaria | 116 |
| 133-M | Induction of quinine resistance in P. falciparum in Aotus monkeys | 119 |
| 134 | Pathogenesis of the malarial paroxysm | 121 |
| 134-A | Evaluation of antimalarial drugs and/or drug regimens | 124 |

| | | |
|---|---|---|
| 134-B | Characterization of new isolates of human malaria ..... | 126 |
| 134-C | Cardiopulmonary changes in malaria in human volunteers .. | 129 |
| 134-D | Limulus assay for endotoxin in experimental malaria ..... | 131 |
| 67-C | Studies on arboviruses other than dengue ..... | 133 |
| 67-D | Studies on the natural history of Toxoplasma gondii, the mechanism of its transmission to man, and its public health importance in the Pacific area..... | 138 |
| 67-H | Studies on dengue ..... | 143 |
| 67-I | Susceptibility and resistance of mosquitoes to dengue ... | 150 |
| 67-J | Observations on a species of Sarcocystis with antigenic, morphologic and biologic similarities to Toxoplasma ..... | 153 |
| 67-K | The susceptibility of Pacific island rats to infection with Angiostrongylus costaricensis ..... | 156 |

Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - July 1, 1972 thru June 30, 1973

## INTRODUCTION

A small loss in total personnel and no significant change in numbers of professional personnel occurred during the year. Departing professionals were Associates Kuberski, Glew, and Knauft and guest worker, Martinez Silva. Associates who arrived in July 1972 were Wyler and Luban. Other arrivals were Staff Fellows, Drs. Robert Gwadz and Dan Harlow. In addition, we were pleased to have Dr. Robert Tesh, formerly of MARU, join the Pacific Research Section. Further details on personnel assignments and foreign travel are provided in the Administrative Report (Project 120) of the Office of the Chief.

The off-site facility of the Laboratory in Hawaii, the Pacific Research Section, is now well established physically within the research and teaching environment of the University of Hawaii Medical School. Although small the professional staff has interests and capabilities in virology, parasitology, and entomology. Operational principles of the Section emphasize utilization of the isolated ecological environments of the Pacific Islands for epidemiological studies and the study of diseases of actual or potential importance in the Pacific area.

The other off-site facility of the Laboratory, the Malaria Units in Atlanta and Chamblee, Georgia has suffered from a chronic attrition of personnel - both professional and technical. In spite of these handicaps productivity of the malaria off-site Units has continued at a high level.

## RESEARCH ACCOMPLISHMENTS

### INCREASING USEFULNESS OF AOTUS MONKEY IN MALARIA RESEARCH

Mosquito transmission: Although human malaria parasites of several species can readily be transmitted and adapted to passage in the Aotus monkey, transmission via infected mosquitoes has generally met with only sporadic success. The group at Chamblee continue to expand their record of successful vector transmissions with both human and simian malarias, including 2 strains of P. vivax and 3 strains of P. falciparum. In addition, the hitherto "impossibility" of infection of Aotus monkeys with P. falciparum from Panama as reported by others was achieved; the key element apparently being the use of Aotus monkeys from Colombia instead of Panama (Collins). Over the past several years all human infections at the human volunteer Unit have been initiated by bite of mosquitoes infected from monkeys (Collins, Miller, Glew and Wyler).

In spite of these encouraging results much remains to be accomplished: More needs to be known about factors which still prevent a reproducible and uniform cyclic transmission of human malaria parasites in the simian host. Other basic problems which can be studied with the simian and vector system includes the study of exoerythrocytic stages of both simian and human parasite species in the liver of the primate host (Collins) and factors affecting gametogenesis and infectivity of the parasite for the mosquito (Gwadz and Collins). One of the new research programs that has been initiated is investigating the genetic influence of Anopheline vectors for susceptibility or resistance to infection with the malaria parasite (Gwadz). Induction of quinine resistance to a strain of P. falciparum in Aotus monkeys is being attempted (Glew).

OTHER BASIC MALARIA RESEARCH UTILIZES PRIMATE MODELS AND VECTORS

Availability of monkey malaria parasites in conjunction with insectary facilities and the background experience of being able to infect mosquitoes has permitted research on the sporogonic cycle in the vector and on the exoerythrocytic stages in the liver of primates. It must be emphasized that considerable experience is required to have both components of the system (the parasite and mosquito) operating in order that experiments can be carried out. Under these circumstances it has been possible to make comparative histological and histochemical studies on parasite development in resistant vs. susceptible mosquito vectors in an effort to learn at what stage development is arrested (Omar and Collins). The effect of various antimalarial drugs given to the infected animals has also been studied upon gametocytes that begin to develop within the mosquito (gametocidal effect) and upon later stages of oocyst and sporozoite development (sporontocidal effect). Compounds which could prevent sporogonic development of plasmodia in the mosquito vector might have practical value in malaria control (Omar and Collins). The hypothesis of Hawking that important circadian changes in infectivity of gametocytes for mosquitoes occurs was confirmed with P. cynomolgi (Powers). The application of chemo-sterilants to female mosquitoes to determine their effect upon malaria parasite development is still another approach to malaria control that is being explored (Omar and Collins). Observations upon complement depletion during the malarial paroxysm in man have led to more detailed study of this phenomenon in the monkey with P. coatneyi malaria in collaboration with workers in LCI (Glew). Systematic studies of the development of exoerythrocytic stages of various species of malaria parasites in the monkey are continuing (Collins).

PATHOLOGY AND IMMUNOLOGY OF MALARIA

Parasite-cell interaction: The interaction of merozoites and red cells is being studied under in vitro conditions that permit direct observation. Treatment of red cell surfaces with various enzymes has given some clues as to the nature of chemical receptors that govern attachment and/or invasion of the parasite (Miller and Dvorak). The distribution and fate of the sporozoite, which is inoculated by the mosquito and initiates infection, is being studied by labeling sporozoites with radioactive isotopes for detection by auto-radiography. Procedures for successful labeling that preserve sporozoite infectivity have been developed (Jacobs and Miller).

Humoral immunity: Interpretation of results with the indirect fluorescent antibody (IFA) test in man after varying time periods and after single or multiple bouts of clinical activity is still not entirely clear. In volunteers IFA titers were often negative by 6 to 8 months and a population in Panama showed a similar pattern 8 months after an epidemic. Nevertheless, utility of the IFA test in assessment of malarial epidemiology during control programs in Central America is being studied in collaboration with CDC (Collins). While some protection to P. knowlesi malaria in monkeys was afforded by immunization this approach using antigens from infected red cells does not appear to be promising (Collins).

Cellular immunity: An increasing effort to understand cellular immune mechanisms in malaria is being made; rosette formation with P. berghei parasites around leukocytes has been demonstrated (Powers and Finerty) and lymphocyte blastogenesis with various antigens of simian malaria is under study (Wyler). The striking specificity of the schizont agglutination test for antigenic variants of P. knowlesi was confirmed, but a modification of the procedure using lysed schizonts resulted in a broadly cross-negative preparation. The significance of these findings to existence of antigens on the surface of the infected red cell vs. antigens on the parasite itself are under further investigation ( Powers and Miller).

MALARIA IN MAN

New treatment: Although total experience is still small, a promising treatment regime has been developed for chloroquine-resistant P. falciparum malaria using combined Clindamycin® and quinine (Miller, Glew, and Wyler). This treatment may have an advantage over other antibiotic combinations using tetracyclines because of shorter duration of therapy and toxicity. In addition, animal studies indicate that if cross-resistance to other commonly used antimalarials develops it does not extend to Clindamycin® (Jacobs). In characterizing behavior of malaria strains from Central America in man resistance to several standard antimalarials was documented for a strain of P. falciparum from Panama, and 2 of 51 infections with P. vivax relapsed after the usually curative 14-day course of primaquine (Miller, Glew and Wyler).

Pathophysiology: Diverse but inter-related studies on malarial pathophysiology have revealed further details concerning the transitory depletion of serum complement (C') that occurs during the paroxysm of P. vivax malaria. Complement-fixing antibody was found to be present and parasitemia of at least several thousand per $mm^3$ occurred in all 11 of 34 paroxysms associated with a significant fall in C'. This would appear to limit C' depletion to later paroxysms during relapse rather than during primary attacks and to rule out complement-mediated processes as a cause of malarial symptomatology (Neva and Glew). In further efforts to understand pathogenesis of the malarial paroxysm transferable pyrogen could not be detected in five patients when 250 to 450 ml of whole blood when given back to the same individual (Neva, Glew and Miller). Also, 22 plasma samples collected from 6 cases of malaria during various periods of their disease were negative for endotoxin-like substances by the Limulus assay (Glew and Levin). A limited investigation of significant blood gas alterations during malarial paroxysms (6 with Falciparum and 1 with Vivax) was negative (Glew and Miller).

BIOCHEMICAL MECHANISMS OF ENERGY METABOLISM

In worms: The ratio of non-heme to heme iron in mitochondria from 2 genera of cestodes exceeds that in mammalian mitochondria. Although an active succinate dehydrogenase was demonstrable in Taenia taeniaeformis a role for this enzyme in terminal electron transport could not be established (Weinbach). Preliminary studies on oxygen utilization by D. immitis microfilariae have indicated that they have an aerobic pattern (Harlow).

In amebae: Two distinct flavo-enzymes have been discovered in E. histolytica which catalyze the oxidation of the pyridine nucleotides NADH and NADPH, respectively. Activity and properties of these two dehydrogenases have been studied (Weinbach). A transhydrogenase which functions in electron transport between NAD and NADP, and which has not previously been reported from protozoa, has been found in E. histolytica. The interaction of this enzyme with the dehydrogenases described above remains to be determined (Harlow). Investigation of adenine nucleotide metabolism of E. histolytica was initiated and has already disclosed that synthesis of ATP from endogenous precursors occurred readily under both aerobic or anaerobic conditions (Takeuchi and Weinbach).

In human blood platelets: Continued studies of platelet metabolism have revealed two quite different types of respiratory activity. Endogenous respiration was completely blocked by inhibitors of mitochondrial metabolism such as cyanide and oligomycin but not by inhibitors of platelet function. In contrast, the thrombin-induced burst of $0_2$ consumption was only partially affected by such mitochondrial inhibitors, but was strongly suppressed by inhibitors of platelet function, such as salicylates (Weinbach and Muenzer).

SCHISTOSOMIASIS

Mortality associated with schistosomiasis in Egypt: In a series of 350 autopsies in Cairo 13% of deaths were attributed to schistosome infection and its immediate complications. Urinary tract obstruction and infection were the principal causes of death, with significant numbers also due to hepatic fibrosis and bladder cancer. Most tissue damage in S. hematobium infections of man and experimental animals is related to an acute diffuse, exudative reaction to eggs, suggesting that delayed hypersenitivity may not be so important in development of lesions. Large numbers of calcified eggs are retained in human tissues but not in experimental animals (Cheever).

Circulating antigen: Since previous workers were able to demonstrate a circulating schistosome antigen in serum of heavily infected animals, a reliable and sensitive assay for the antigen would permit a new approach in quantifying severity of infection. A method was developed to partially purify the worm antigen and this was coupled to methylated bovine serum albumin to produce a precipitating antiserum, a crucial reagent for further work. The purification procedure plus an array of physiochemical tests indicate that the antigen is a large molecular weight polysaccharide. Antibody to the antigen has been detected in the serum only of lightly infected cases. With now available knowledge of the nature of the antigen and improved reagents for its detection, work on developing a radio-immunoassay is going forward

as well as studies on distribution of the antigen (Nash and Neva).

Genetic work on snail vectors: Studies on the genetics of susceptibility have continued, comparing infections in two different species of snails, Biomphalaria glabrata and B. straminea, with two different Carribbean strains of S. mansoni. Juvenile snails of the two species show the same 3 genetic susceptibility types, suggesting the basic mechanism is probably the same. Several types of tumors occurring in stocks of the snail, B. glabrata, have been under study and appear to be genetically determined. Histopathology of these lesions is also under investigation. Incidental observations on parasites of snails or obscure pathology continue to be made (Richards).

AMERICAN TRYPANOSOMIASIS

Cell-parasite interaction in in vitro chamber: Parasite and environmental factors were found to influence the lag period and doubling time of the intracellular cycle of T. cruzi. The intracellular doubling time of infective stages obtained directly from infected bugs was found to be about 8 hours and this period progressively lengthened to 11 or 12 hours after passage in cell culture (Luban and Dvorak). The effect of incubation temperature was found to be more complex in the beef cell system used. With increasing temperatures from 29° to 38°C, the lag period prior to onset of reproduction decreases. However, as temperature increases from 32° to 38°C the time for reproduction (doubling-time) increases so that the net effect of increasing temperature tended to be zero. "Beating" myocardial cells which are infected with T. cruzi continue to display this functional activity throughout the reproductive phase of the parasite within them (Dvorak).

T. cruzi antigens: Further evidence was found that the cross-reactions of cell culture antigen by complement fixation (CF) with some sera of patients with American leishmaniasis are due to excessive contamination with beef cells. In addition, sera from some cases of American leishmaniasis were reactive by CF with beef antigen alone. Preliminary results of gel-diffusion analysis indicate that cell culture antigens have unique characteristics as well as shared components with the traditional epimastigote antigen (Neva).

Tissue tropism of different strains of T. cruzi: The most prominent histopathologic feature in mice infected with two strains of vector-passaged T. cruzi was the prominence of lesions in skeletal muscles. Yet, one strain produced high parasitemia while the other had few circulating parasites. In contrast, a blood-passaged strain produced parasitic foci and extensive damage in the liver. After one passage in vector bugs, this latter strain no longer multiplied in the liver (Mercado).

TOXOPLASMA AND RELATED ORGANISMS

Toxo-like organism in the cat: Another interesting but confusing chapter to the Toxoplasma story has been uncovered by the finding of a "new" organism that produces oocysts in the cat which are indistinguishable from those of Toxoplasma. The oocysts of this "new" organism

when inoculated into mice develop into sarcocysts in the muscle and hence are presumed to be Sarcocystis muris. This organism shares some antigenic, morphologic and biologic properties with Toxoplasma and may turn out to have some implications insofar as human infection is concerned (Wallace).

More on Toxoplasma in cats: Further evidence is accumulating from the Pacific region for the concept that felids are essential hosts in maintaining Toxoplasma in nature and that infections in humans and wild rodents are related to presence of cats (Wallace). Yet, more details of the role of the cat in epidemiology of this infection are needed, such as the influence of immunity upon excretion of the infective stage in the feces. With at least one strain of Toxoplasma, cats that are re-infected within 6 months of a previous infection do not generally excrete oocysts. However, this pattern may depend upon whether the prior infecting strain produces oocysts (Sheffield).

Diagnosis and drug treatment of toxoplasmosis: A serologic test based upon staining of organisms with horseradish peroxidase that has been coupled to anti-human IgG is being evaluated for toxoplasmosis as well as for other parasitic infections (Lunde). The effect of drugs upon oocyst production in infected cats and upon morphology of the parasite in vitro is being studied (Sheffield). Chlorinated lincomycin analogues, which are effective in treating acute disease in mice, did not have a therapeutic effect upon the cyst stage in chronically infected animals (Lunde and Powers).

AMEBIASIS

Latest on the amebal viruses: All strains of amebae (total of 9), including one of the presumably avirulent E. hartmanni, which have been tested have yielded a virus. All but one have been of the polyhedral type but even these do not seem to be entirely of the same variety on the basis of their host-range for different strains of amebae. The nucleic acids of all the viruses, including the filamentous variety, appears to be composed of DNA and the DNA strands of the polyhedral agent exhibit a unique orderly arrangement by electron microscopy. The fact that 27 individual clones of amebae that were selected in an effort to find a virus-free clone all showed evidence of carrying the virus is rather strong evidence that the polyhedral virus exists in a lysogenic state with its host cells (Diamond, Mattern and Hruska).

Amebic disease in man and animals: Past experience in this Laboratory and elsewhere has led to the concept that axenically cultivated E. histolytica, regardless of origin, becomes avirulent for experimental animals. The work of Sepulveda from Mexico indicating that axenically grown amebae produce lesions when inoculated directly into the hamster liver has been confirmed and extended. Even with this technique strains vary in virulence. Another laboratory rodent, the jird, has also been found to be susceptible. This new tool for study of amebae is being utilized for further studies. The amebiasis skin test using histolyticin in a graded dose range from axenically-grown amebae has been evaluated in 450 individuals in India. These plus earlier studies indicated that the optimum dose which will not induce sensitization to the antigen and give fewest false positive reactions but still be active

is 6 micrograms of amebic protein (Diamond, Phillips and Indian PL 480 workers).

FILARIASIS

Occult filariasis: This condition of presumed filarial infection without circulating microfilariae (mf) being demonstrable is still incompletely understood. When an experimental model for the disease is created in dogs by immunizing them vs. mf, serum from the animals will cause agglutination of mf. The mf agglutination reaction is affected by labile serum components, including complement. Full specificity of the reaction has not yet been established (Knauft and Pacheco). Sera from well-characterized cases of eosinophilic lung collected in Singapore 10 years ago were found to show striking elevations of serum IgE (mean of 4980 mgms %) in comparison with patent filariasis cases (784 mgm % mean). Preliminary results indicate that IgE levels drop quite promptly after therapy (Pacheco and Kaplan, LCI). Comparative response of cats, dogs, jirds, and Patas monkeys was studied to single and repeated infections with Brugia malayi filaria. No obvious difference in microfilaremia patterns were evident in single as compared to multiple infections, but the jird had higher and longer lasting microfilaremia (Pacheco).

Serologic tests in filariasis: In cooperation with the U.S. State Department the performance of a Dirofilarial antigen has been compared in the soluble antigen fluorescent antibody (SAFA) test with the same antigen as used in an indirect hemagglutination (IHA) test at NIH. Each test had its own unique problems in regard to specificity and neither appeared to be reliable for use in diagnosis of human filarial infections (Pacheco and Wolfe, State Department).

Action of drugs in filariasis: Because diethylcarbamazine (DEC) is effective in clearance of microfilariae (mf) only in vivo and has no in vitro effect vs. mf it seemed that immune status of the host could be uniquely evaluated on the basis to response to DEC. This was tested in dogs given 4 different biologic exposures to D. immitis. In the dog D. immitis system, however, microfilaremia response was the same in all groups, regardless of immunologic status, and relatively large doses of DEC were found necessary to affect mf (Pacheco and Knauft). Mebendazole®, an anthelminthic vs. nematodes and cestodes and manufactured in Europe was tested for antifilarial activity in jirds infected with D. viteae. A 10-day treatment with 10 mgm/kg twice daily by gavage was effective vs. adult worms as judged by loss of mf in most treated animals and greatly reduced recovery of adult worms after treatment (Pacheco).

BIOCHEMICAL AND GENETIC STUDIES ON NEMATODES

Genetics of development: A genetic "map" has been constructed for the free-living nematode, Caenorhabditis elegans, mainly from mutants with developmental defects of growth, molting and behavior. Nutritional and growth mutants affected during the third molt stage are the most prevalent. A large number of genes located in separate linkage groups can affect the same phenotype.

For instance, short mutant isolates which approximate the third molt juvenile stage in length are mapped at over 20 locations in 5 separate linkage groups. The third juvenile molt stage is frequently implicated in the infective processes of parasitic nematodes (Pertel).

Cytochemical and structural studies on the contact chemoreceptor: The contact chemoreceptor of the nematode has been postulated to be the amphid on the basis of structural and genetic studies. The ultrastructure and cytochemistry of the nerves which comprise the sensory network of C. elegans was described (Mattern and Pertel).

STUDIES ON DENGUE FEVER

Is hemorrhagic dengue due to "sensitization"? Investigation of a recent outbreak of dengue on the remote island of Niue in the South Pacific raises new questions about the etiology and pathogenesis of severe and fatal dengue. Prior to this study it was widely believed that hemorrhagic dengue was the result of "sensitization" due to repeated infection with different dengue serotypes. On Niue it was concluded that most of the severe and fatal cases followed a first infection with the virus. This would favor the hypothesis that dengue virus strains, like those of poliomyelitis, may vary in their virulence. If this is the case, the possibility of severe forms of dengue occurring in Hawaii, the Caribbean, and the southern United States is greater than previously believed (Rosen).

Further advances in dengue lab tests: Two significant advances in laboratory techniques for work with dengue viruses were (a) development of a method to use the CF antigen produced in mosquitoes after direct inoculation to type and therefore identify dengue viruses, and (b) the use of a direct fluorescent antibody technique to detect infected mosquitoes. These procedures, along with the previously discovered method using male mosquitoes to isolate, titrate, and produce high-titered pools of dengue virus, greatly improve the armamentarium for work with what was previously considered a "difficult" group of viruses (Rosen and Kuberski).

STUDIES ON ARBOVIRUSES

New work initiated: Studies on other arthropod-born viruses of known or potential importance in the Pacific area has been started. Some of the new techniques recently developed for study of dengue viruses are being tested out for possible usefulness on other arboviruses such as Chickungunya and the sand fly-associated viruses. Some promising results have already been obtained (Tesh).

Serial No. NIAID 120
1. Parasitic Diseases
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Administration from the Office of the Chief

Previous Serial Number: Same

Principal Investigator: Franklin A. Neva

Other Investigator: Eugene C. Weinbach (Acting Assistant Chief)

Cooperating Units: Section on Clinical Parasitology, Laboratory of Clinical Investigation, NIAID

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 45/12 | | | 45/12 |
| Professional | 7/12 | | | 7/12 |
| Other | 38/12 | | | 38/12 |

Project Description:

This project furnishes administrative and supervisory services to research projects in the Laboratory as follows:

Research plans are discussed with various investigators and overall research activities are coordinated. Travel of personnel is arranged and secretarial services are provided. Assistance is given in the preparation, editing, and reviewing of manuscripts and reports. Requisitioning and supervision of equipment and supplies and maintenance of the library is provided. Consultation and the liaison activities are maintained with individuals, academic organizations, and other branches of the Federal Government in matters pertaining to the activities of the Laboratory. Arrangements are made for supervision of research workers who are in a training status. Centralized facilities and supervision are provided for care of experimental animals used by investigators.

Liaison is maintained with the Section on Clinical Parasitology in the Laboratory of Clinical Investigation with reference to diagnostic services or special investigations that are carried out on patients at the Clinical Center. Dr. Theodore Nash, one of the Clinical Associates from LCI is continuing his research project on schistosomiasis in this Laboratory but maintains certain clinical responsibilities and assignments as a member of LCI.

Further reorganization of the malaria research program has continued. The immediate effect will be a cessation of induced infections in prisoner volunteers and a phase-out of the Unit on Human Malaria at the Federal Penitentiary in Atlanta, Georgia during the coming fiscal year. Reasons for this decision were multiple, but the action helps in continuing an active research program in malaria with increased emphasis upon primate models and the insect vector.

The Pacific Research Section in Honolulu is now firmly esconced in its new and well-designed quarters at Leahi Hospital of the University of Hawaii Medical School. The facilities were visited and officially opened by former NIH Director, Robert Marston in August, 1972. Fairly regular telephone communications on a weekly basis via space satellite between Bethesda and Honolulu continues. A collaborative project with Dr. Chaniotis, formerly of MARU in Panama, but now working temporarily at Rocky Mountain Laboratory, and with Dr. Rosen on mosquito susceptibility to dengue viruses has been initiated. Robert Tesh, M.D., a virologist-epidemiologist formerly at MARU, joined the Pacific Research Section in January, 1972.

Three Research Associates are finishing their two years of work with us - Dr. Timothy Kuberski of Pacific Research Section, Dr. Richard Glew of the Unit on Human Malaria, and Dr. R. Frederic Knauft of the Bethesda Laboratory. Newly arrived Associates in July 1972 included Dr. David Wyler at Atlanta, Georgia and Dr. Norman Luban in Bethesda, Maryland. Dr. Martinez-Silva, a guest worker, left to take a new position in November, 1972. Dr. Omar continues with us as a Visiting Associate in Chamblee, Georgia, as does Dr. Duane Gubler in Honolulu. Dr. Allen Cheever is still on special assignment in Cairo, Egypt until July 1973. The only other change in professional staff was the arrival of Dr. Robert Gwadz, an entomologist, as Staff Fellow in July, 1972 to work with the malaria group in Chamblee, Georgia.

Foreign travel in connection with research work during FY 1973 included:

Drs. W.E. Collins, Robert Gwadz and L.H. Miller to San Salvador, El Salvador, Central America.

Dr. Guillermo Pacheco to Santo Domingo and San Pedro de Macoris, Dominican Republic.

Dr. L.S. Diamond and Mr. M.N. Lunde to Europe and India on Dr. Diamond's PL-480 project; Dr. L.S. Diamond to Mexico City, Mexico.

Dr. Leon Rosen and Dr. Timothy Kuberski to American Samoa and Dr. Rosen to additional sites in the Far East and Pacific too numerous to mention.

Dr. Louis Diamond continued to administer the visiting guest lecturer arrangements the past year and Dr. Weinbach provided strong back-up as Acting Assistant Laboratory Chief.

Honors and Awards:

F. A. Neva

Appointed Lecturer in Tropical Public Health at Harvard University School of Public Health, Boston, Massachusetts.

Continued to serve as representative of American Scoeity of Tropical Medicine and Hygiene on the National Council for International Health, with membership on several sub-committees of this Council.

Member of Advisory Board of Gorgas Memorial Institute of Tropical and Preventive Medicine, Inc.

Invited Lectures, Conferences, or Talks Given:

1. Harvard School of Public Health on Chagas' disease, Oct., 1972.

2. Series of lectures on parasitology at University of Kentucky, Lexington, Kentucky, Oct., 1972.

3. Seminar on Chagas' disease at Johns Hopkins University School of Hygiene and Public Health, Department of Epidemiology, Baltimore, Maryland, Nov., 1972.

4. Invited participant in Workshop on Schistosome Antigens sponsored by Naval Medical Research Institute, Bethesda, Maryland, Mar., 1973.

5. Invited participant in Workshop on Trypanosomiasis and Leishmaniasis sponsored by the Walter Reed Army Institute of Research, Washington, D.C., Apr., 1973.

6. Visiting Professor at Duke University Medical School, Durham, North Carolina, Apr., 1973. Gave 2 lectures and made ward rounds with several different infectious disease groups.

Publications:

Neva, F.A. Parasitic Diseases of the GI Tract in the United States. Disease-a-Month, June 1972.

Serial No. NIAID 121-C
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The biology and control of the snail intermediate hosts of schistosomes

Previous Serial Number: Same

Principal Investigator: Charles S. Richards

Other Investigators: None

Cooperating Units: Dr. Edward H. Michelson, Department of Tropical Public Health, School of Public Health, Harvard University.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 24/12 | | | 24/12 |
| Professional | 12/12 | | | 12/12 |
| Other | 12/12 | | | 12/12 |

Project Description:

Objectives: To study molluscan genetics with particular attention to applications to control measures. To study prospective agents for biological control of intermediate hosts of the schistosomes. To study the biology of the snails with the aim of improving the efficiency of control measures. To study the contributions and complications of molluscan diseases and molluscan genetics to bio-medical research. To study the causes of tumors in mollusks.

Methods Employed: Selection and crossing experiments are conducted to find additional genetic markers, to characterize linkage groups, to increase knowledge of mechanisms of molluscan genetics, and to investigate further the genetic aspects of molluscan susceptibility to infection and to molluscicides.

Parasites of mollusks are studied to determine: their identity, molluscan host range, mode of transmission, localization and pathology of infection, and interrelations with schistosome infections. Electron microscopy is employed in studies on protozoa and bacteria.

Genetic studies, fresh dissections, and stained serial sections are employed to determine the causes and pathology of tumors in mollusks.

Major Findings: Studies on the genetics of susceptibility of B. glabrata for infection with S. mansoni were continued. Snail stocks of four of the seven genetic susceptibility types observed, with respect to Puerto Rican and St. Lucia strains of S. mansoni, have been established for use as test stocks. Using these test stocks results indicated a strain of S. mansoni from Ethiopia was similar to the St. Lucia strain, while a strain from Tanzania was different from the above strains, being less infective.

Attempted crosses between susceptible B. glabrata from Dominican Republic and other stocks of B. glabrata indicated they were relatively incompatible. Descendents of one of the few $F_1$s obtained have been crossed with refractory stock snails. Selection and testing of hybrid offspring suggests the probability that refractory incompatible stocks can be developed.

Snails of a species of Biomphalaria from the island of Grenada were exposed to S. mansoni miracidia. None of the adults but 7% of the juveniles exposed became infected, and some shed cercariae, demonstrating the fact that the species was a potential intermediate host for S. mansoni. Although these snails appear similar morphologically to Biomphalaria straminea, they differ in some minor features. Several attempts to cross the Grenada Biomphalaria with B. straminea failed, and species identification remains to be determined.

Crosses have been established in mice between Puerto Rican and St. Lucia strains of S. mansoni by exposures to cercariae from snails infected by single miracidia from either of the trematode strains. Both male Puerto Rican X female St. Lucia, and female Puerto Rican X male St. Lucia crosses were obtained. B. glabrata from our genetic test stocks have been exposed to $F_1$ miracidia.

Studies on the genetics of B. straminea were continued for comparison with B. glabrata. Exposures of juvenile B. straminea to Puerto Rican and St. Lucia S. mansoni has revealed the same three susceptibility types as in B. glabrata: refractory to both, susceptible to both, or susceptible to Puerto Rican but refractory to St. Lucia S. mansoni. True breeding stocks of B. straminea of the first two types have been established. Hybrid $F_1$s of a cross between snails of these two types tested like the refractory parent.

Studies on the histopathology of tumors, genetic and otherwise, in B. glabrata are being carried out with the collaboration of Dr. E. H. Michelson, School of Public Health, Harvard University. A polypoid type of tumor has been observed on the mantle collar of several B. glabrata in one clonal stock in frequencies indicating inheritance. Another type of tumor, observed in frequencies suggesting either inheritance or parent to offspring transmission of an infectious agent, is associated with the digestive gland but becomes detached and grows in the hemocele as an unattached tumor with fingerlike projections. Another abnormality involving masses of cells believed to be hemacytes has been observed in the auricle of a number of B. glabrata, in frequencies suggesting transmission of either genetic factors or infectious agents.

Studies on protozoan and bacterial parasites of snails were continued. The severe pathology involving shell mat formation in B. glabrata infected with the microsporidan Steinhausia brachynema ( = Coccospora brachynema, Richards & Sheffield, 1970), shown to be the result of an inherited host-parasite relation, has also been observed in B. straminea.

Lesions were observed in a stock of B. glabrata from Belo Horizonte, Brazil. Beginning as internal cysts usually in the side of the foot, these lesions open to the outside after a few days emitting strings of mucus and dead cells. Later the lesion becomes an empty flask-shaped cavity with small circular opening to the outside. Variable percentages of juvenile progeny associated with infected parents were infected. Attempts to infect other stocks of B. glabrata by contact with infected snails of this stock have failed. The causitive agent has not been determined, but results indicate that susceptibility to infection is inherited.

Significance to Bio-medical Research and the Program of the Institute:

Establishing stocks of B. glabrata, of various combinations of juvenile and adult susceptibility to strains of S. mansoni differing in infectivity, provides material for bio-chemical, biological and cytogenetic intraspecific analyses of the basic mechanisms of susceptibility.

Need for evaluation of the role of genetics in snail control continues. Refractory B. glabrata may prove useful in biological control of schistosomiasis by dilution or replacement of populations of susceptible snails in endemic areas. Progress in understanding causes of susceptibility or insusceptibility of B. glabrata to infection with S. mansoni accelerates the prospect of safe field testing of refractory snail stocks for control. Development of a refractory stock of B. glabrata incompatible with the endemic snail population in a problem area may avoid dilution of the introduced insusceptibility by interbreeding.

Establishing markers for some of the various linkage groups in B. glabrata increases our capacity to analyze invisible and complex genetic characters such as host-parasite relations.

The monogenetic three allele pigment character in B. straminea provides a genetic marker enabling studies of the genetics of susceptibility in that snail species for comparison with B. glabrata.

Information (including histopathology) on a variety of tumors observed in B. glabrata, many of which are genetic, should help in alayses of tumors of mollusks collected in the field.

Microsporida, amebae, and bacteria cause extensive damage in some snail hosts. These and other snail diseases need to be examined for potential human infection, and prospective use as agents in biological control of snails. It is essential to be aware of the occurrence of these infections and their relations with the snails and other parasites to plan, conduct, and interpret experimental research on snails with validity.

Results increasingly implicate genetics in host-parasite relations involving various parasites.

Proposed Course of the Project: Genetic studies on B. glabrata will be continued to: (a) seek additional genetic markers; (b) expand our knowledge of linkage groups; (c) characterize and establish true breeding stocks of the various susceptibility combinations of B. glabrata for S. mansoni infections; (d) test additional stocks of B. glabrata and strains of S. mansoni as available and (3) continue studies of incompatibility between certain stocks of B. glabrata.

Preliminary tests suggest that a strain of S. mansoni from Tanzania is progressively less infective than strains we have tested from Puerto Rico and St. Lucia. Tests will be continued to characterize the infectivity of the Tanzania strain and to establish true breeding stocks of the additional genetic susceptibility type of B. glabrata differentiated by this S. mansoni strain.

After the results of crosses in mice between strains of S. mansoni from Puerto Rico and St. Lucia are analyzed, crosses will be made to obtain $F_2$ and backcross information.

Comparison of the genetics of susceptibility in other snail species such as B. straminea with B. glabrata will continued.

Studies on protozoan and bacterial infections in mollusks will be continued: including relations to the molluscan hosts, effects on other parasites, potential for human infection, and as a variable factor in experimental research. Studies will be continued on the lesions in the Belo Horizonte, Brazil, B. glabrata to identify the infectious agent involved, and to clarify the role of genetics in susceptibility to infection.

Molluscan tumors will be studied by fresh dissections and culturing, serial sections, genetic selection and crossing, and environmental

manipulation to determine causal factors. Studies on the histopathology of molluscan tumors will be continued in collaboration with D. Michelson.

Honors and Awards:

Participation in symposium on "Molluscan Genetics" at the annual meeting of the American Malacological Union, Incorporated, July 10-14, 1972, Galveston, Texas.

Participation in Mollusk Seminar, University of Michigan, Ann Arbor, Michigan, August 25-26, 1972.

Participation in Workshop on "Pathologic Effects of Chemicals on Aquatic Organisms" sponsored by Environmental Protection Agency, Pensacola, Florida, May 8-9, 1973.

Publications:

Richards, C. S.: Biomphalaria glabrata genetics: Pearl formation. J. Invertebrate Pathology, 20: 37-40, 1972.

Richards, C. S. and Merritt, J. W., Jr.: Genetic factors in the susceptibility of juvenile Biomphalaria glabrata to Schistosoma mansoni infection. Am. J. Trop. Med. Hyg., 21: 425-434, 1972.

Richards, C. S.: A potential intermediate host of Schistosoma mansoni in Grenada. J. Parasit., 59: 111, 1973.

Richards, C. S.: Pigmentation variations in Biomphalaria glabrata and other Planorbidae. Malacol. Rev., 6: 49-51, 1973.

Richards, C. S.: Susceptibility of adult Biomphalaria glabrata to Schistosoma mansoni infection. Am. J. Trop. Med. & Hyg., (In Press).

Richards, C. S.: Genetics of Biomphalaria glabrata. Malacol. Rev. (In Press).

Serial No. NIAID 121-H
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Pathogenesis of Schistosome Infections in Mammalian Hosts

Previous Serial Number: Same

Principal Investigator: Allen W. Cheever
(Detail assignment U.S. Naval Medical Research Unit No. 3, Cairo, Egypt - 7/1/71 - 6/30/73)

Other Investigator: None

Cooperating Units: Department of Medical Zoology, Walter Reed Army Institute of Research (Drs. Sadun and Erickson); Department of Pathology, Peter Bent Brigham Hospital, Boston, Mass. (Drs. von Lichtenberg and Smith); Department of Parasitology, Southwest Foundation, San Antonio, Texas (Drs. Kuntz and Myers); East Africa Institute for Medical Research (Dr. Eyakuze); Departments of Pathology, Cairo University Medical School (Drs. Elwi and Kamel) and Ein Shams University (Dr. Soliman), Cairo, Egypt; Naval Medical Research Unit No. 3, Cairo, Egypt; Tanta Mission Hospital, Tanta, Egypt; Department of Urology, Cairo University Medical School (Dr. Torky); Department of Pathology, U. Bahia, Brazil (Dr. Andrade)

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 16/12 | | | 16/12 |
| Professional | 12/12 | | | 12/12 |
| Other | 4/12 | | | 4/12 |

Project Description:

Objectives: The interactions between various mammalian hosts and schistosomes of medical importance are studied, particularly the dynamics

of egg production by the parasite and the excretion or destruction of eggs by the host. Pathologic lesions are studied in relation to the above variables and to physiologic changes, such as portal hypertension and hepatic and gastrointestinal function. Infected persons are studied using quantitative techniques to determine the role of intensity of infection in producing clinically significant disease, to evaluate the differences in disease seen in different geographic areas and to provide baseline data against which the relevance of animal models can be measured.

Methods Employed: Laboratory rodents and primates infected with S. mansoni, S. haematobium or S. japonicum are studied during the course of infection and are sacrificed at various intervals after infection. Pathological and parasitological findings at autopsy are correlated with observations before death. Surgical specimens from schistosome infected patients are studied to correlate pathological changes with the numbers of eggs present in the tissue. Autopsy cases are examined to relate the presence and intensity of schistosome infection to the lesions noted.

Major Findings: About 10% of deaths in 350 autopsy cases studied are attributed to S. haematobium infection. Obstructive uropathy and acute and chronic pyelonephritis were the cause of death in 8% of cases and carcinoma of the bladder in 3%. About 3% of deaths are attributed to S. mansoni infection, in which hepatic fibrosis and portal hypertension were the cause of death. With both schistosome species, death from schistosomiasis was much more frequent in heavily infected cases.

S. haematobium eggs are widely distributed in other organs, particularly the colon and lungs, but do not appear to produce appreciable pathology outside the genitourinary system. S. haematobium infection occasionally produces severe schistosomal colonic polyposis, but the great majority of such cases are related to S. mansoni infection. Although it is a frequent cause of severe morbidity, colonic polyposis was the cause of death in only 1 case.

The reaction to S. haematobium eggs in man and experimental animals is basically similar in the acute phase, with both diffuse exudative and granulomatous response to live eggs. Fibrosis frequently follows this phase in both man and animals. Thereafter, the process differs radically, with persistence of large masses of calcified eggs in man, while in animals, eggs are destroyed by host reaction. In both man and experimental animals, the diffuse exudative reaction is associated with much more damage to host tissues than is the granulomatous reaction, suggesting that delayed hypersensitivity is not the principal pathogenetic mechanism.

Patients with carcinoma of the bladder generally have much more intense S. haematobium infections than do other cases.

Significance to Biomedical Research and the Program of the Institute:

The relation of significant disease in schistosome infected individuals to the intensity of schistosome infection is clear in our material. These and other data indicate that lightly infected individuals are not at great risk, and clinical management and control programs should take this relative risk into account. In Egypt, there can be no doubt that schistosomiasis is a major cause of morbidity and mortality.

In addition to allowing comparison of the intensity of schistosome infection in different geographic areas, quantitative study provides insight into the comparison of infections in man and experimental animals. Infections in animals show much more tissue damage in relation to the numbers of eggs present because of the rapid removal of eggs from the tissues following the phase of maximal reaction to eggs.

Proposed Course of the Project: Most of the next year will be devoted to analysis of the data gathered in the past two years. Talapoin monkeys will be infected with S. haematobium. This species developed bladder cancer when infected with S. haematobium, as did more readily available capuchin monkeys. The latter have failed to develop genito-urinary lesions regularly, and more extensive testing of talapoins is necessary. The relation of hepatic portal lesions to glomerulonephritis in schistosome infection is being investigated in chimpanzees. Pulmonary disease, most not caused by schistosome infection, will be compared in autopsy material from Egypt and the U.S.A.

Honors and Awards:

Dr. Cheever served on the Council of the American Society of Tropical Medicine and Hygiene, was an invited participant at the meeting on classification of bladder cancer of the National Bladder Cancer Project, and a faculty member in the American College of Physicians Postgraduate Course on Problems of International Health.

Publications:

Anderson, L.A. and Cheever, A.W.: Comparison of geographic strains of Schistosoma mansoni in the mouse. Bull. Wld. Hlth. Organ. 46: 233-242, 1972.

Powers, K.G. and Cheever, A.W.: Comparison of geographic strains of Schistosoma mansoni in the rhesus monkey. Bull. Wld. Hlth. Organ. 46: 295-300, 1972.

Cheever, A.W. and Powers, K.G.: Schistosoma mansoni infection in rhesus monkeys: comparison of the course of heavy and light infections. Bull. Wld. Hlth. Organ. 46: 301-309, 1972.

Cheever, A.W.: Pipestem fibrosis of the liver. Trans. Roy. Soc. Trop. Med. Hyg. 66: 946-948, 1972.

Cheever, A.W., Erickson, D.G., Sadun, E.H. and von Lichtenberg, F. Schistosoma japonicum infection in monkeys and baboons: parasitological and pathological findings. Am. J. Trop. Med. Hyg. (in press).

Soliman, L.A.M., Cheever, A.W., Kuntz, R.E. and Myers, B.J. Lesions of bladder muscle in baboons and monkeys infected with S. haematobium. Trans. Roy. Soc. Trop. Med. Hyg. (in press).

Cheever, A.W., Kuntz, R.E., Myers, B.J., Moore, J.A. and Huang, T.C. Schistosomiasis haematobia in african, hamadryas and gelada baboons. Am. J. Trop. Med. Hyg. (in press).

Serial No. NIAID 121-I
1. Parasitic Diseases
2. Host Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Serologic Studies on Filariasis

Previous Serial Number: Same

Principal Investigator: Guillermo Pacheco

Other Investigators: Anastasia Stanley and R. Frederic Knauft

Cooperating Units: Dr. Thomas C. Orihel, Delta Regional Primate Center, Covington, Louisiana. Dr. Angel M. Ponce, San Pedro de Macoris, Dominican Republic. Dr. Martin S. Wolfe, Department of State, Washington, D.C.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 20/12 | | | 20/12 |
| Professional | 18/12 | | | 18/12 |
| Other | 2/12 | | | 2/12 |

Project Description:

Objectives: To study serologic changes in experimental and natural infections with various filariae in man and animals. Antigens from different stages in the life cycle of homologous or heterologous parasites are used in these studies.

Methods Employed: Indirect tanned-cell hemagglutination (IHA) is used as the basic technic to evaluate humoral antibody response. Fluorescent antibody technics are used to demonstrate antigen-antibody complexes with soluble antigens; diffusion in agar and immunoelectrophoresis are used as adjuncts in analysis of antigens.

Sera used in the above tests are from: (a) jirds, dogs, cats or Patas monkeys infected once or periodically with Brugia malayi, (b) jirds, dogs or cats infected once or periodically with B. pahangi, (c) Patas monkeys infected with Dirofilaria corynodes, (d) dogs experimentally infected with Dirofilaria immitis, or noninfected dogs immunized with D. immitis microfilariae (MF) (resulting in the inability of homologous to circulate upon subsequent infusion of the parasite), (e) sera from U.S. citizens returning to the United States suspected or proved to have infections with filariae - Onchocerca volvulus, Loa loa, Dipetalonema perstans - (f) sera from families in San Pedro de Macoris, Dominican Republic, in which one or more members have microfilaremia of Wuchereria bancrofti, (g) sera obtained from patients in the Clinical Center with either leukemia or lymphoma, and (h) sera from rabbits either immunized with Freund's complete adjuvant and metabolic products of MF of D. immitis, B. malayi, or W. bancrofti or given several infusions of living MF of D. immitis or B. malayi

Antigens used for serologic tests are: (a) fractionated extracts of adult male and female D. immitis, (b) metabolic products of third-stage infective larvae of Dipetalonema viteae, B. malayi or B. pahangi or (c) metabolic products of microfilariae of D. immitis, B. malayi, B. pahangi, or W. bancrofti.

Major Findings: An extract of adult D. immitis, routinely used as antigen for IHA in this laboratory, was given to Dr. Martin Wolfe and Mr. William McCartin of the Medical Laboratory, U.S. Department of State, for use in a soluble-antigen fluorescent-antibody (SAFA) test to determine whether the SAFA test would provide similar or different results than IHA in tests of the same sera. Sera tested were from: (a) people from Dominican Republic with or without microfilaremia of W. bancrofti, (b) patients from the NIH Clinical Center with leukemia or solid tumors of various types, (c) U.S. citizens, employed by Department of State, that had not been out of the U.S., (d) U.S. citizens suspected or proved to have other filarial infections - Onchocerca volvulus, Loa loa or Dipetalonema perstans and (e) dogs experimentally infected with D. immitis. Results indicated clearly that despite use of the same worm extract the two tests detected different antigen-antibody systems. However, despite detection of different antigen-antibody systems, antibody was not detected by either test in many people with microfilaremia. False positive reactions were common in IHA tests of sera from patients with leukemia only (30%), these were not observed by SAFA. By contrast, sera from many laboratory-reared uninfected dogs reacted strongly in the SAFA test and not at all in IHA. High antibody titers were detected by both tests in sera from patients with clinical filariasis without microfilaremia. It seems clear from these, as well as previous, studies that antigens derived from whole adult D. immitis have limited utility in diagnosis of human nonclinical filariasis.

The serologic response of cats, dogs, monkeys, and jirds exposed to either single or multiple fortnightly reinfections of third stage Brugia malayi larvae has been determined and compared. There was no difference in peak titer or duration of titer between singly or reinfected animals. The jird differs from the other three hosts as sera tested with extracts of adult D. immitis had high antibody titers 2 weeks after infection which persisted well past stabilization of microfilaremia. In the other hosts a lower antibody titer was detected early in infection which decreased coincident with appearance of microfilaremia and disappeared shortly after patency.

A method of separating MF from blood was devised which permits an efficient, > 90%, recovery of MF washed free of plasma and cellular components. Large numbers, 1-25 million, of D. immitis and B. malayi MF have been collected and incubated for various lengths of time. Metabolic products have been concentrated and combined with Freund's adjuvant to immunize rabbits. Serum from dogs in which infections with D. immitis were prepatent, patent with different levels of microfilaremia, or post-patent have been tested by gel diffusion and immunoelectrophoresis against the rabbit anti-microfilarial metabolic products globulin. This work is still in progress but at least 4 different antigen-antibody systems are present in dogs with microfilaremia, 2 in pre- and post-patent dogs, and more than 2 in noninfected dogs immunized with D. immitis in which homologous MF do not circulate.

Significance to Bio-medical Research and the Program of the Institute:

Adequate evaluation of currently available serologic tests used in the diagnosis of filariasis has proved difficult because of lack of sera from controlled infections as well as ignorance of changes that may occur in levels of antibody with respect to time. The comparative study of IHA with SAFA recognized these problems by testing sera from patients in an endemic area of Bancroftian filariasis in conjunction with sera from controlled animal infections. The SAFA test is not more specific than IHA and neither test is useful to detect nonclinical filariasis.

Serologic diagnosis remains inadequate because of lack of specificity and the inability of the group of antigens present in extracts of adult D. immitis to provide results which would be meaningful in the spectrum of filarial diseases. Previous work with metabolic products of third-stage larvae as antigen indicated specificity and sensitivity in reinfected animals. Present work, with metabolic products of MF may contribute a useful antigen(s) to detect occult infection or disease associated with MF when these are undetectable in peripheral circulation.

Proposed Course of Project: Attempts to define the existing antigen-antibody systems present in rabbits immunized with D. immitis MF will be carried out as well as comparing the system present in infected dogs with dogs in which MF do not circulate. It is hoped that this information can then be applied to a similar model for B. malayi and W. bancrofti, filarial

parasites of man. Additionally it is hoped that continued contact with San Pedro de Macoris, Dominican Republic, can be maintained to regularly obtain W. bancrofti MF as well as follow people in an endemic area to learn more about changes in antibody levels over time.

Serial No. NIAID 121-J
1. Parasitic Diseases
2. Host-Parasite Relationship
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Host-parasite relationships in filariasis

Previous Serial Number: Same

Principal Investigator: Guillermo Pacheco

Other Investigators: Dr. R. Frederick Knauft, Research Associate, PHS

Cooperating Units: Dr. Thomas C. Orihel, Delta Regional Primate Center, Covington, Louisiana; Dr. Angel M. Ponce, San Pedro de Macoris, Dominican Republic, Dr. Allen Kaplan, LCI, NIAID; Dr. Glen Geelhoed, NCI.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 28/12 | | | 28/12 |
| Professional | 18/12 | | | 18/12 |
| Other | 10/12 | | | 10/12 |

Project Description:

Objectives: To study the interaction between filariae and their vertebrate hosts. These studies are directed to provide a better understanding of filariasis and other nonpatent helminthiases in man.

Methods Employed: Brugia malayi, B. pahangi, Dipetalonema viteae, Dirofilaria immitis and D. corynodes, as well as appropriate arthropod vectors, are maintained for experimental study. Blood and tissues from monkeys infected with D. corynodes or B. malayi, and people infected with Wuchereria bancrofti are received from collaborating laboratories.

To study the effects of various stages of filarial life cycle, as well as possible interaction of these stages, vertebrates are given: (1) third-stage larvae from an appropriate arthropod vector, (2) adult worms by

subcutaneous or intravascular surgical implantation or (3) washed microfilariae (MF) by intravenous infusion. Numbers of leucocytes, eosinophils and, when present, MF are estimated at preset intervals. When vertebrate hosts are necropsied representative samples of tissue as well as the adult worms that are recovered are preserved for later study.

Major Findings:

A. Infection with filariae: To compare basic parameters of host and parasite response to infection with Brugia malayi groups of cats, dogs, jirds, and Patas monkeys were given single or fortnightly infections of preset numbers of larvae. The duration of the prepatent period in dogs, cats or monkeys which developed microfilaremia was similar to the average for the jird, 20 weeks, with a range of 14-26 weeks. Microfilaremia was significantly higher in reinfected jirds. Maximum eosinophilia was seen in single or reinfected jirds 6 weeks after the first injection of larvae. Thereafter, regardless of reinfection, eosinophilia decreased and was indistinguishable from that of uninfected jirds by 20 weeks. This pattern was also seen in cats but statistically, there was no difference in eosinophilia between infected, reinfected, or uninfected monkeys or dogs. Infection in the jird differed from the other hosts in two striking ways: microfilaremia was both considerably higher and longer lasting and antibody, detected by IHA, persisted at high levels throughout patency. These studies are still in progress, primarily to determine duration of microfilaremia and histopathologic changes.

B. Mode of Action of Diethylcarbamazine: work in Japan with Litomosoides carinii in cotton rats indicated that the immunologic status of the host determined whether MF would be cleared following treatment with diethylcarbamazine (DEC). A microfilaricidal effect of the drug was seen only in cotton rats with high levels of antibody demonstrable by IHA. To determine whether the same phenomena are operative with other filariae, dogs with D. immitis were treated daily with increasing doses of DEC. Effect of DEC on MF was monitored in dogs prepared with four biologically different exposures to the parasite. (1) Infective larvae maturing to adult worms producing MF (2) MF only - dogs received infusions of MF from an infected donor dog (3) transplanted mature worms producing microfilaremia and (4) infective larvae were prevented from maturing by prophylactic administration of DEC. Later MF were infused into these animals. Antibody responses in the 4 groups, as measured by IHA, were predictably different, yet the response of MF to the drug was similar. Microfilaremia in all groups irrespective of drug dose was suppressed within 5 minutes, but with low doses 5-50 mgm/kg recovered to pretreatment levels 8 hours after drug administration. Sustained suppression of microfilaremia occurred in all groups when treated with 200 mgm/kg for 5 days. Sustained suppression was characterized by a descending stair case effect, i.e. the same pattern of suppression--recovery, seen with low doses of DEC, was present but on successive days the level of recovery was less than on the preceding day. Previous work demonstrated that the lung is a major resevoir of MF, that only a small

percentage of the total population of MF in a host are in peripheral circulation. To determine whether the reduction in microfilaremia evoked by DEC occurred only in peripheral circulation and to monitor the effect of the drug on the population of MF in lung, catheters were surgically placed in pulmonary artery and aorta. Microfilaremia was monitored from the two sites and a peripheral vein simultaneously after drug administration. The marked reduction in microfilaremia was similar in the three implying that the drug does not induce a regional redistribution of MF but most likely causes sequestration of MF in capillary circulation.

C. Occult filariasis: Dogs have been made immune to D. immitis MF by repeated intravenous infusion of living MF. Thereafter homologous MF fail to circulate when injected and are destroyed in lung. This phenomenon is coincident with the appearance of an in vitro agglutination of MF when these are incubated in immune serum. This reaction has the following characteristics: It is abolished by reduction of serum with 2-mercapto-ethanol and by heating serum at 56°C for 30 minutes. The latter is restored by adding guinea pig serum as a source of complement. The reaction seems specific although Brugia pahangi from cat agglutinates in immune serum, but this reaction is not affected by heating at 56°C. Factors other than complement appear to be labile as agglutination does not occur in serum stored at 25°C for 5 days even when guinea pig serum was added. In vitro adhesion of leukocytes to MF has also been investigated and is dependent on immune serum. Studies are in progress to further characterize and compare the factors responsible for agglutination and adhesion and to determine the cell type(s) involved with adhesion and the consequences of the reaction on cells and MF.

D. Evaluation of mebendazole as an antifilarial chemotherapeutic drug: Mebendazole, ethyl 5-benzoylbenzimedazole-2-carbamate, developed by Janssen Pharmaceutica, Belgium, may be a broad spectrum anthelmintic as data indicates it is effective against several nematodes and cestodes important to man. It has not been extensively evaluated against filariae, but a small trial in Indonesia suggested clinical efficiency. A pilot study testing the drug against Dipetelonema viteae in the jird has been completed. After infections were patent, 10 mgm/kg of drug was given by gavage twice daily for 10 days to infected and uninfected animals. Microfilaremia after 5 days of treatment was reduced and disappeared by the 10th day. Microfilaremia reappeared in one treated animal. The animals were sacrificed 10 days after end of therapy. Adult worms were recovered from all untreated animals, but only one gravid female was recovered from treated animals, the one animal in which microfilaremia recurred. Leuckocytosis was not observed in any of the jirds at any time. While not remarkable, eosinophilia in infected jirds was usually over 5% and quite variable, often changing from 6% to 15% from one week to the next. This pattern of variability persisted throughout the period of observation. By contrast, eosinophilia in infected jirds that were treated decreased gradually after the first week of treatment until at the end of the period of observation it was at an essentially normal level - 1 or 2%.

E. IgE levels in filariasis: Sera from patients in Singapore collected in 1962 at a clinic specializing in tropical pulmonary eosinophilia (TPE) and from people with bancroftian filariasis in the Dominican Republic have been analyzed for total immunoglobulins including IgE. Levels of IgG, IgA and IgM were unremarkable in either group but IgE was impressively elevated in patients with TPE compared to other form of filariasis, mean values of 4,980 mg% vs. 784 (normal $\leq$ 600). This work is in progress but a comparison of IHA, eosinophilia, and IgE levels in TPE and changes in these parameters during and after treatment with DEC are planned. Preliminary results indicate that IgE decreases sharply one month after therapy.

Significance to Bio-medical Research and the Program of the Institute:

Basic interaction of host and filariae are poorly understood. The availability of the jird as a model, in which two of the filariae that parasitize man would develop provides an opportunity to learn more about these interactions. However comparing infection by Brugia species in jird, dog, cat and monkey indicates that the latter two species may more accurately reflect what is seen in man.

Although DEC has been the major drug in the management of filariasis, its mode of action remains unknown. Comparing results of drug effect in the dog-D. immitis and cotton rat-L. carinii models suggests that there are two phases to its action. The first, common to both systems, may be a pharmacologic effect in which MF are sequestered in capillaries and the second, manifest by sustained suppression of microfilaremiae in the cotton rat, might reflect an immunologic mechanism leading to destruction of MF. The latter may be facilitated by the sequestration. Further clarification of factors responsible for the agglutination of MF and cellular adhesion in immune sera may indicate the biological significance of these events. Better understanding of the immune mechanism may provide insight into one type of immune response which may be related to the mode of action of DEC.

It is well accepted that TPE is associated with the death of MF in lung, however the pathogenesis and probable immunopathogenesis of the disease remains unexplained. The role of immediate hypersensitivity hopefully can be investigated in the near future now that techniques to obtain significant numbers of MF their metabolic products, and assay systems measuring pharmacologic substances released by lung passively sensitized by sera from patients with TPE are available.

Chemotherapy of filariasis remains inadequate because of side effects of present drugs used and some species of filariae seem unaffected by drugs currently available, i.e. Dipetalomena perstans. Careful clinical trials of mebendazole in addition to further investigation of the drug against laboratory models of Brugia Dipetalonema sp. are warranted.

Proposed Course of the Project:

Continuing studies on filariasis will emphasize the effects of reinfection in various hosts with human filariae. Special attention will be given to establishment of models in which occult filariasis and immediate hypersensitivity can be studied.

Honors and Awards:

Dr. Pacheco was an invited participant in the U.S.-Japan Cooperative Medical Science Program, Parasitic Disease Conference held in Hiroshima, Japan in July 1972; organized and chaired a Workship on Filariasis for the Tropical Medicine and Parasitology Study Section of DRG, NIH. This Workshop was held at Tulane University, New Orleans, Louisiana, in September, 1972; gave lectures in Parasitology to first-year medical students at the University of Pittsburgh School of Medicine and continued participation in the Tropical Medicine and Parasitology Study Section, DRG, NIH.

Publications:

Pacheco, G., Atkins, J. and Gurian, J. 1972. Quantification of infection of ticks with Dipetalonema viteae. J. Parasit. 58: 275-278.

Tulloch, G.S., Pacheco, G., Casey, H.W., and Anderson, R.A. 1972. Diethylcarbamazine chemoprophylaxis for canine dirofilariasis induced by multiple infections. Canine Heartworm Disease: The Current Knowledge. Gainesville. Univ. of Florida Press. pp. 109-111. Ed. R.E. Bradley and G. Pacheco.

Tulloch, G.D., Pacheco, G., Anderson, R.A., and Miller, F.H., Jr. 1972. Scanning electron microscopy of adult male Dirofilaria immitis. Canine Heartworm Disease: The Current Knowledge. Eds. R.E. Bradley and G. Pacheco. Gainesville. Univ. of Florida Press. pp. 113-115.

Serial No. NIAID 121-K
1. Parasitic Diseases
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Detection and characterization of a circulating schistosome antigen and other immunologic studies in schistosomiasis.

Previous Serial Number: None

Principal Investigators: Theodore E. Nash and Franklin A. Neva

Other Investigators: Benjamin Prescott

Cooperating Units: Howard Sloan (NHI)

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 18/12 | | | 18/12 |
| Professional | 12/12 | | | 12/12 |
| Other | 6/12 | | | 6/12 |

Project Description:

Objectives: 1. To characterize the nature of the circulating antigen 2. To purify this antigen and develop a sensitive enough assay system so that amounts present in the circulation of man can be detected. 3. To determine the location of the antigen in the schistosome. 4. To define the response of host to the antigen.

Methods Employed: Antibodies to this antigen have been raised in rabbits immunized with a mixture of methylated bovine serum albumin and partially purified antigen. This antisera reacts specifically with the circulating antigen but does not react with normal mouse or normal human sera. The antigen is detected using immunodiffusion, Ouchterlony, or counter-immunoelectrophoresis. The antigenic activity resides only in the trichloroacetic acid soluble-chloroform insoluble fraction of homogenized worms. This fraction is used for further immunization, purification and as substrate for various enzymatic and degradative procedures.

Major Findings: The antigenic activity does not reside in the protein or lipid fraction of worm homogenates but in the trichloroacetic acid soluble-chloroform insoluble fraction. This is predominantly carbohydrate with less than 1% nitrogen present. The antigenic activity remains unaltered after exposure to pronase, trypsin, and $\alpha$ amylase, DNase, RNase, and alkaline phosphatase. Sialic acid is not present in the active fraction, nor is deoxyribose or ribose. Activity is destroyed with sodium metaperiodate. Sepharose 4B chromatography of the whole worm homogenate demonstrates that activity is present in all but the last protein peak. It is therefore most likely a large molecular weight polysaccharide. Using sephadex DEAE, the antigen is present in the most tightly bound fraction but can be eluted off with high ionic strength solutions. Antibodies to the antigen were found in three of eight patients with clinical schistosomiasis.

The antigenic carbohydrate fraction was activated with cyanogen bromide to which tryamine was attached. Using this procedure about 12 to 35 tyramine molecules can be attached to every 250,000 molecular weight units. The tyraminated antigenic fraction is then labelled with $I_{125}$. About 10% of the material is precipitated by immune antisera. Further purification is needed to develop a viable radio-immune assay.

Significance to Bio-medical Research and the Program of the Institute:

It has recently been shown that significant disease in schistosomiasis varies directly with worm burden. Quantitative eggs counts offer a rough estimation of worm burden, however measurement of excretion and/or secrection products in the host's circulation offers a means of quantification, diagnosis, and possibly prognosis. The role that this particular antigen plays in the worm metabolism remains unexplored and could lead to new chemotherapeutic approaches. The host's response to the antigen also remains unexplored. Particularly interesting is the possibility of locating antigen in the glomeruli and portal tracts of patients suffering from glomerulonephritis and Symmers' fibrosis.

Proposed Course of the Project: Purification of the antigen will enable the development of a sensitive radio-immune assay and further characterize the molecular nature of the antigen. It is expected that this assay will detect antigen in the circulation of man and possibly lead to an estimation of the worm burden. Immunofluorescent techniques will be used to locate the antigen from the worm and host tissues.

Honors and Awards:

Participant, Schistosome Antigen Conference, March 1973, Naval Research Institute, Bethesda, Maryland.

Serial No. NIAID 122-G
1. Parasitic Diseases
2. Parasite Growth and Differentiation Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Entamoeba histolytica and other parasitic protozoa

Previous Serial Number: Same

Principal Investigator: Louis S. Diamond

Other Investigators: Carl F. T. Mattern, J. Hruska, and Bruce P. Phillips

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 39/12 | | | 39/12 |
| Professional | 8/12 | | | 8/12 |
| Other | 31/12 | | | 31/12 |

Project Description:

Objectives: To develop and refine techniques for the axenic cultivation of Entamoeba histolytica, related amebae and other parasitic protozoa; to define their in vitro nutritional requirements, to study their metabolism, and mechanisms of pathogenicity. To ascertain factors involved in cyst production of Entamoeba. To study naturally occurring viruses of E. histolytica. To develop techniques for cryopreservation of parasitic protozoa, to study mechanisms involved in resistance of these organisms to freezing injury.

Methods Employed: Entamoebae or other parasitic protozoa freed of naturally associated microorganisms by use of microisolation procedures and/or a variety of antibiotics are established in monoxenic cultures with single species of trypanosomatids or bacteria, then adapted to grow under axenic conditions. In vitro growth is monitored through use of a Coulter electronic counter and/or standard counting chambers.

Axenically cultivated E. invadens is exposed to crithidial lysates and the process of the ensuing encystment of the amebae studied by electron microscopy.

Viruses of E. histolytica are characterized and studied using classical virological techniques.

Protozoa are suspended in a variety of crypopreservatives (glycerol, dimethylsulfoxide, glucose, etc.) and the effects of varying freezing and thawing rates on recovery of viable cells cooled to temperature of liquid nitrogen (-196°C) are studied.

Laboratory animals are inoculated via laparotomy with amebae cultivated under a variety of conditions and pathogenesis is studied.

Major Findings: Study of the recently discovered viruses of Entamoeba histolytica continues as the principal research effort of this laboratory. Since the last report, polyhedral virus has been detected in two additional strains of these amebae bringing to 9 the total number of strains examined and found infected. No additional instances of infection with the filamentous virus have been uncovered.

A polyhedral virus morphologically similar to the ones identified in E. histolytica and capable of producing CPE in this species has been detected in a strain of the relatively innocuous parasite of man, E. hartmanni.

The nature of the nucleic acids of the polyhedral agent from strain HB-301:NIH and the filamentous virus from HM-1:IMSS E. histolytica amebae has been investigated. Studies with the metabolic inhibitors bromodeoxyuridine, idodeoxyuridine and cytosine arabinoside suggest that the nucleic acid of both viral types are composed of DNA. Furthermore, the nuclear inclusions produced by the filamentous virus fluoresced a chartreuse color with acridine orange, while homology studies with the nucleic acid of the polyhedral virus provided additional evidence that this virus was composed of DNA.

The polyhedral virus of E. histolytica has been shown to be icosahedral in shape. Its folded DNA strand has been found to have a unique orderly, packing arrangement.

HK-9 amebae infected with HB-301 polyhedral virus show a 3- to 5- fold post-infection increase in DNA synthesis. DNA to DNA hybridization studies with this newly synthesized DNA and DNA from uninfected amebae show the former to be primarily viral in nature.

In isotope incorporation studies using tritiated thymidine most of the radioactivity was found associated with the nuclear fraction of strain HK-9 amebae infected with the filamentous agent. Conversely, in the case of infection of the same strain with the polyhedral agent most of the activity

was in the cytoplasmic fraction. These findings were consistent with EM studies showing the former virus developing in the nucleus and the latter in the perinuclear cytoplasm (Diamond, Mattern and Hruska).

Twenty-seven clones, representing two generations, of HB-301 amebae have been assayed for indigenous polyhedral virus and found infected. These results along with other evidence previously reported tend to rule out the possibility that the ameba-virus association is a simple carrier system, and strengthens the concept of a lysogenic system.

Investigations performed in this laboratory and elsewhere (Wittner and Rosenbaum, Albert Einstein College of Medicine, New York) have shown that virulent strains of E. histolytica rapidly become avirulent in axenic culture judged by their ability to colonize and produce lesions after intracecal inoculation into conventionally reared guinea pigs. Recently, Sepulveda and colleagues (Centro de Estudios Sobre Amibiasis, Mexico) showed that these "avirulent" amebae (HK-9 strain) produce lesions in hamster livers when inoculated intrahepatically. Sepulveda's work has been confirmed and extended in this laboratory. Not only can it be shown that the axenically reared amebae are capable of producing lesions in the hamster liver, but also that strains vary in virulence as they do when cultivated conventionally (i.e. with a wild mixed bacterial flora). Moreover, the Jird has been shown for the first time to be susceptible to amebic infection. Intrahepatic inocuation of the amebae leads to production of lesions in the liver.

During the course of these animal experiments, amebae taken from infected livers have been re-established directly in axenic culture. This apparently marks the first time that E. histolytica has been axenized directly from infected animals without recourse first to the use of microbial associates to establish the cultures (Diamond and Phillips).

The fine structure of the trophozoite, precyst and cyst stages of E. invadens from axenic cultures have been studied. A hitherto undescribed structure has been observed in the trophozoite and precyst but not the cyst (Diamond and Mattern).

Significance to Bio-medical Research and the Program of the Institute:

The ameba-virus association provides a relatively simple system for the investigation of a virus in a primitive erkaryote and knowledge gained from this system should enhance our overall understanding of viral-mammalian cell relationships.

New approaches in the study of hepatic amebiasis can be made now with the development of a method for assaying virulence of axenically cultivated E. histolytica in hamsters and jirds. The technique should be particularly useful for studying the possible relations of the amebal viruses to amebic disease.

The axenic cultures of E. histolytica developed in this laboratory continue to provide the basic research tool for studies of the immunology, metabolism, and molecular biology of this important parasite of man (see Project Nos. NIAID 123-C, 125-K and 125-P).

Proposed Course of the Project: Efforts will be made to further characterize the physical and chemical properties of the viruses of Entamoeba. A classification of these viruses based on host-range specificity is being developed. A third generation of amebal clones has been initiated and will be examined for presence of polyhedral virus. Avirulent strains of E. histolytica have been obtained and are in the process of being assayed for virus. The search for viruses in other species of Entamoeba as well as other pathogenic protozoa will continue. The possible role of amebal viruses in hepatic amebiasis will be investigated using the hamster and jird animal models. Patients with amebic disease are being screened serologically for antibodies against amebal viruses. Electron microscopic studies of encystation in E. invadens will be continued. Efforts to develop techniques for the axenic cultivation of other parasitic protozoa will continue, as will efforts to improve existing techniques for the cryo-preservation of these organisms.

Honors and Awards:

Chairman, Advisory Committee to the Collection of Protozoa, American Type Culture Collection.

Re-appointed to Board of Trustees and elected to Executive Council of the Board, American Type Culture Collection.

Re-appointed to WHO Expert Advisory Panel on Parasitic Diseases (General Parasitology).

Member, Executive Committee, Society of Protozoologists.

Member, Editorial Board, Cryobiology.

Invited Conference Leader, Fifth Seminar on Amebiasis, sponsored by National University of Mexico and the Mexican Institute of Social Security, Mexico City, February 21 to 23.

Named as one of five founding members of the Centro de Estudios Sobre Amibiasis, Mexico City.

Publications:

Phillips, B. P., Diamond, L. S., Bartgis, I. L. and Stuppler, S. A. 1972. Results of intracecal inoculation of germfree and conventional guinea pigs and germfree rats with axenically cultivated Entamoeba histolytica. J. Protozool. 19: 498-499.

Hruska, J. F., Mattern, C. F. T., Diamond, L. S. 1973. Viruses of Entamoeba histolytica. III. Properties of the polyhedral virus of the HB-301 strain. J. Virol. 11: 129-136.

Serial No. NIAID 122-J
1. Parasitic Diseases
2. Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

**Project Title:** Biochemical Basis for Growth and Differentiation of Parasitic and Free-Living Nematodes

**Previous Serial Number:** Same

**Principal Investigator:** Dr. Ruth Pertel

**Other Investigators:** Dr. Carl F.T. Mattern, Laboratory of Viral Diseases

**Cooperating Units:** None

**Man Years:**

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 24/12 | | | 24/12 |
| Professional | 12/12 | | | 12/12 |
| Other | 12/12 | | | 12/12 |

**Project Description:**

Objectives: To investigate the generalized life cycle of parasitic and free-living nematodes utilizing techniques from biochemistry, genetics, and neurobiology. To investigate sensory modulation in the wild type of C. elegans and in its mutants at various stages of differentiation and development in an attempt to elucidate the sensory mechanism involved in the transitional phase of nematodes when a free-living mode is exchanged for a parasitic mode and the development of the reproductive system begins.

To develop techniques of investigating neuronal network synaptic contacts, and morphogenesis of the gonads in the large number of mutants altered in behavior and postembryonic development of the transitional phase.

Methods Employed: Free-living Caenorhabditis elegans var. Bristol cultured as synchronous populations under axenic conditions (free of all living associates) are used as the initial test system. Chemotactic, motility, drug resistant, and developmental mutants are used to study the

neuronal basis for the responses of nematodes to their environment which controls reproduction. Developmental mutants are studied in regard to their genetics, population dynamics, and media effects upon growth and development.

Chemotactic assay: A biphasic (senisolid agar and aqueous system) is used to determine the sensitivities of various mutants to chemicals.

Anatomical studies: Conventional techniques of electron and light microscopy are used.

Major Findings:

1. Anatomical studies: a) The fine structure of the neuronal sensory structures involved in chemoreception have been described (Mattern and Pertel). b) Synaptic markers were determined for papillary and amphidial nerves using cytochemical techniques for acetylcholinesterase, choline acetylase, and the catacholomines. A flourescent dansylated bungarotoxin was used to locate sites of acetylcholine reception in the sensory synapses. (Pertel). c) Feulgen staining of whole mounts of C. elegans was used to detect gross changes such as cell deletions from tissue systems. The ventral nerve cord in a class of short mutants designated "dumpy" were found to contain fewer nuclei than the expected normal number of 60 in the wild type. These worms develop normal gonads but do not increase in length beyond the third to fourth juvenile molt stage. This indicates a relationship between the nerve cells that failed to undergo mitosis post embryonically and attainment of full adult size. The genetic control for this growth affects the important transitional stage. Mutants of this type were mapped on five of the six chromosomes. (Pertel)

2. Genetics: Six linkage groups representing the six chromosomes have been established. Genetic mapping of 300 mutants is underway. Approximately 220 isolates have been mapped into 128 separate genes.

a) The holding stage (ensheathed dauerlarva) of parasitic nematodes prior to infection of the host usually occurs at some point prior to the development of the gonads. In the Strongyles and in C. elegans ( a putative evolutionary relative of the Strongyles), development of the gonads begins at the third to fourth stage molt transition of the juveniles. We are interested in the degree that genetic vs. environmental information affects this transition. Deletion from the growth medium of hemin and steroid, essential nutrients of C. elegans, produced environmentally determined holding stages, i.e., growth stoppage with maintenance of a particular molt stage and even halted reproduction in the wild type adult. However, in approximately 30 mutants, mapped as separate genes, the third to fourth juvenile molt stage was the one uniformly affected in terms of growth and developmental phenomena. Growth and anatomical studies are continuing on these mutants. (Pertel)

b) Dauer modification temperature-sensitive mutants: Initially a mutant was described which retained its second stage cuticle and which manifested a life cycle dependency upon a change of medium after it had gone through a reproductive cycle and growth to the third stage. Behavioral differences were also evident in pre-third stage larvae which were no longer apparent in the adult. Specific media components which would reinitiate growth at this stage were determined. Conditional temperature-sensitive nutants of this type have now been isolated as well as further isolates of the first type. (Pertel)

3. Protein, DNA and cAMP assays of synchronized populations of axenically grown C. elegans have been made in order to determine a synchronization index for monitoring populations to be used in future developmental studies. The DNA was constant in the somatic tissue. Protein and cAMP levels increase at the same rate. (Pertel)

Significance to Bio-medical Research and the Program of the Institute:

Nematode infections of man, domestic animals, and plants are protean in character and world-wide in distribution. The studies on the mechanism of sensory processing of environmental information by nematodes would provide information on how nematode parasites find their hosts and specific tissues in the host.

The nematode system also offers a good study system for the understanding of the basic biology of differentiation and development. It is an in vivo system that uniquely separates the differentiation of one tissue, the gonad, from other fully differentiated tissues. Most other systems for studying the differentiation of the blast stages of a tissue are in vitro cell cultures. These systems are plagued with contamination by transforming viruses which change the genetic characteristics of the cells, and by gene dosage effects when the chromosomes undergo abnormal ploidy changes. The information derived from the nematode studies will also provide understanding of the basic interactions attendant upon the development of parasitic nematodes as influenced by the host which so often occurs at the third to fourth molt stage transition when the gonad develops and the primary biological business of reproducing new parasites begins.

Proposed Course of the Project: The environmental stimuli required to initiate the development of the gonads, and the sensory modulation of this stimuli information will be pursued by further studies with a variety of sensory and developmental mutants. The mapping of the developing nervous system and the effects of the nervous system on the developing gonad of the nematode will be continued at the light and electron microscope level. In addition, the study of the neurotransmitter characteristics of junctions in the anterior end of the worm will be continued.

In situ hybridization of the various DNA's will be performed to locate the chromosomal or cytoplasmic origin of the various satellite and redundant DNAs. The function of specific DNAs eliminated at the various stages of development will be pursued.

Honors and Awards:

R. Pertel, Instructor, Behavioral Genetics of a Nematode, Cold Spring Harbor Laboratory, Long Island, N.Y. Aug. 2-19, 1972.

Chair person - Symposium on "Model Systems for the Study of Behavior and Development in Nematodes". Society of Nematologists Annual Meeting, Raleigh, North Carolina, Aug. 4, 1972.

Chair person - Workshop on "Genetics and Aging in Nematodes", NICHD, Cold Spring Harbor Laboratory, Long Island, N.Y. Aug. 20-22, 1972.

Participant, Workshop on "Nematode Cell Culture", NICHD, Hunt Valley, Maryland, Feb. 12-14, 1973.

Invited speaker on the following occasions:

"Behavioral Genetics of a Nematode", Laboratory of Biochemical Genetics, NHLI, NIH, July 28, 1972.

"Nematode Chemotaxis", Section on Neuroendocrinology, NHLI, July 12, 1972.

"Axenic techniques for mutant selection", NICHD Workshop on Nematode Genetics and Aging. Aug. 21-22, 1972.

"Postembryonic Developmental Mutants of C. elegans", Helminthological Society of Washington, Jan. 19, 1973.

"Behavioral Genetics of a Nematode", Dept. of Biology, University of New Hampshire, Durham Feb. 24, 1973.

"Postembryonic Developmental Mutants of a Nematode" Medical and Graduate Faculty Luncheon, University of Louisville Mar. 23, 1973.

"Post embryonic Developmental and Behavioral Mutants of a Nematode". Dept. of Developmental Biology, State University of New York at Stony Brook, May 17, 1973.

"Behavioral Mutants of C. elegans". Invited Symposium speaker, American Society of Parasitology meetings, June 27-30, 1973, Toronto, Canada.

Publications:

Wilson, S., and Pertel, R. Histamine Content of Neuroblastoma. Science (In Press).

Pertel, R., and Wilson, S. Histamine Content of the Nematode,

Caenorhabditis elegans. Comp. Biochem. Phy. (In Press).

Pertel, R., and Gershon, D. Selective Methods for Mutants of the Axenically Grown Nematode, Caenorhabditis elegans. Proc. Nat. Acad. Sci. (In Press).

Pertel, R. Genetic Analysis of Induced Mutants of Caenorhabditis elegans. Genetics. (In Press).

Serial No. NIAID 123-B
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Pathological physiology and histochemistry of parasitic diseases

Previous Serial Number: Same

Principal Investigator: Teresa I. Mercado

Other Investigators: None

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 24/12 | | | 24/12 |
| Professional | 12/12 | | | 12/12 |
| Other | 12/12 | | | 12/12 |

Project Description:

Objectives: To gain a better understanding of the pathological physiology of parasitic diseases by physiological and histochemical studies.

To examine differences between various strains of Trypanosoma cruzi by studying the susceptibility of host tissues to parasitic invasion and assessing the alteration of certain parameters.

Methods Employed: Tissues from mice infected with Trypanosoma cruzi strains of different origins (vector-derived and blood-passaged) are studied by means of standard histological procedures, staining with Giemsa, and histochemical assaying for lipid and glycogen. Parasite levels of inocula and progression of parasitemia are determined by hemocytometer counts. Brain, liver, and muscle, including cardiac, skeletal, from gastrocnemius, rectus abdominis, and diaphragm, and smooth from gastro-intestinal tract, were studied. The following strains were examined: (1) House 510 (vector-derived), House 33 (vector-derived), Tulahuen (vector-derived), and Tulahuen (blood-passaged).

Major Findings: Strain House 510: As early as 12 days after inoculation, although parasitemia levels were minimal, parasitic foci and changes in skeletal muscle occurred; a moderate proliferation of the connective tissue surrounding the muscle bundles was observed. After 17 days (parasitemias ranging from 17 to 26 millions per ml. of blood) muscle deterioration was quite extensive particularly of gastrocnemius. Wide separations of individual muscle bundles resulted from the massive proliferation of connective tissue. The rectus abdominis muscle showed marked multiplication of parasitic foci rather than the extensive deterioration described above. Diaphragm appeared to be the least susceptible muscle. Cardiac and smooth muscles were moderately parasitized. Longer periods of infection (up to 40 days) and parasitemias ranging from 23 to 42 millions caused further deterioration of muscle including diaphragm which at this period of infection showed a marked increase in the occurrence of parasitic foci and displacement of muscle fibers. No parasites were observed in liver even during high parasitemias but aggregates of large mononuclear cells were observed in the region of the bile ducts and in small areas throughout the lobule. In the more advanced infections necrosis occurred. Lipid infiltration was marked but there was no significant glycogen depletion. Parasitization of brain was observed after 23 days of infection.

Strain House 33: The most significant finding observed with this infection was the lack of parasitemia. Even after 37 days of infection the parasite count did not rise above 4 trypanosomes per 30 microscopic fields. This strain appeared to be more infective to skeletal muscle than to cardiac or smooth muscle. Extensive involvement was observed after 22 and 37 days of infection and, as was observed with the House 510 strain, diaphragm required the longest period of infection to exhibit significant changes. Liver changes were comparable to those described above, parasites being conspicuously absent.

Tulahuen Strain (blood-passaged): Essentially a reticulotropic strain; numerous parasitic foci occurred in Kupffer cells throughout the liver lobule and large areas became necrotic after 7 to 11 days of infection. Lipid infiltration occurred as early as 3 days after inoculation and progressed rapidly, large accumulations commonly occurring after 7 days. Marked glycogen depletion accompanied the lipid infiltration, a finding which is in marked contrast to that obtained in the vector-derived infections. However, as in the latter, connective tissue proliferation in the region of the bile ducts was observed, sometimes being quite intense. The changes observed in muscle were considerably less significant than those produced by the House 510 and House 33 strains.

Tulahuen Strain (vector-derived): The most significant observation made with this strain was its characteristic absence from the liver, a finding which was comparable to that obtained with the other vector-derived strains but in marked contrast to the blood-passaged Tulahuen strain from which it was derived. Parasitemias as high as 6 millions per ml. of blood were obtained after 24 days of infection. Liver changes were also comparable to those observed with the House 510 and House 33 strains.

Brain involvement occurred only with the House 510 strain.

In view of the liver changes observed in the vector-derived infections, although no parasitic foci were present, the possibility was examined that some factor from the vector, other than the parasite, was involved. Accordingly, gut contents from 9 uninfected Rhodnius prolixus were pooled and inoculated into 5 normal mice. No tissue changes were observed and levels of lipid and glycogen were normal.

Significance to Biomedical Research and the Program of the Institute:

Basic cellular studies provide a means for understanding the physiological mechanisms involved in the response of the host to parasitic infections and may lead to the identification of specific parasite substances involved in pathogenesis. Histochemical studies aid in localizing the altered sites within tissues. Physiological studies quantitate the alteration of activity observed histochemically and aid in defining further the physiological mechanisms that may be damaged. Basic studies on strain differentiation aid in taxonomic classification and in assessing the pathogenicity of a parasitic infection.

Proposed Course of the Project: Additional strains of Trypanosoma cruzi will be studied. Future research on strain differentiation will also include biochemical studies on serum isoenzyme levels of parasitized mice.

Publications:

Mercado, T. I.: Calcium in the liver of mice infected with Trypanosoma cruzi. J. Parasit. 58: 1137-1142, 1972.

Mercado, T. I.: Inhibition by splenectomy of a paralyzing syndrome in rats infected with Plasmodium berghei. Exp. Parasit. (In press).

Serial No. NIAID 123-C
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biochemical mechanisms of energy metabolism in mammalian and parasitic organisms.

Previous Serial Number: Same

Principal Investigator: Eugene C. Weinbach

Other Investigators: Louis H. Diamond, Dan Harlow (Staff Fellow), and Tsutomu Takeuchi (Visiting Fellow)

Cooperating Units: Laboratory of Microbiology, NIAID (Theodore S. Theodore) (Project No. 63B), Department of Biochemistry, Case Western Reserve University, School of Medicine (Joseph Muenzer)

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 35/12 | | 6/12 | 41/12 |
| Professional | 21/12 | | 6/12 | 27/12 |
| Other | 14/12 | | | 14/12 |

Project Description:

Objectives: To conduct fundamental studies on the mechanism of aerobic energy metabolism in mammalian and in parasitic organisms. Specifically: To continue studies of electron transport and oxidative phosphorylation in mammalian cells and mitochondria, and (B) comparative studies with cells and mitochondria from parasites.

Methods Employed: Animal tissues are fragmented by mechanical or enzymatic disruption of the cells and mitochondrial fractions are isolated by differential centrifugation. Similar procedures are used to isolate mitochondria from cestodes, the principal metazoan parasites used in current studies.

Entamoeba histolytica, strain HK-9, is cultivated axenically in Diamond's liquid medium, and the active trophozoites collected from 72-hours cultures by centrifugation. For diaphorase studies, the trophozoites are sonicated and the soluble fraction obtained after centrifugation is used in spectrophotometric and polarographic assays. Enrichment of the diaphorase activities is achieved by acetone precipitation and polyacrylamide disc gel electrophoresis.

Human platelets are isolated from freshly drawn whole blood by differential centrifugation.

Mesosomal vesicles and plasma membranes are isolated from Staphlococcus aureus by differential and sucrose density gradient centrifugation after treatment of the bacteria with lytic enzymes.

Cellular and mitochondrial constituents are determined and characterized by appropriate enzymatic, chemical and physical methods. Oxygen consumption is determined polarographically with the Clark oxygen electrode. Enzyme activities are assayed by spectrophotometric and radionuclide methods.

Major Findings:

A. Parasite Studies

1. Cestode mitochondria: Continued studies on the role of non-heme iron in the cestode respiratory chain provided new evidence that the ratio of non-heme to heme iron in cestode mitochondria exceeds that of mammalian mitochondria. Direct chemical analyses disclosed that this ratio approaches 6 in mitochondria isolated from Taenia taeniaeformis, Hymenolepis diminuta and Hymenolepis microstoma.

It was reported previously that although succinate oxidase activity was not demonstrable in mitochondria of T. taeniaeformis, succinate elicits a characteristic non-heme iron signal which was detected in the electron paramagnetic resonance spectrometer. Spectrophotometric assays with T. taeniaeformis mitochondria and ferricyanide as electron acceptor confirmed the presence of an active succinate dehydrogenase. The specific activity of the cestode dehydrogenase is about one-half that found in rat liver mitochondria. The parasite enzyme was inhibited by malonate and 2-thenoyltrifluoracetone, a potent chelator of non-heme iron. In cestode mitochondria succinate dehydrogenase was 10-fold more sensitive to the iron chelator than was glycerol-3-phosphate dehydrogenase, an enzyme that is linked to terminal electron transport and oxygen utilization in this parasite.

2. Diaphorase activities in Entamoeba histolytica: Continued studies on the biochemistry of E. histolytica disclosed that this parasitic protozoan has the enzymatic capacity to catalyze the oxidation of both reduced pyridine nucleotides, NADH and NADPH. We have shown by several

basis of comparison for determination of adenine and pyridine nucleotides, and for testing various enzyme assays. Such studies were necessary to insure that the methodology employed with parasites were optimal and reproducible.

2. Energy metabolism in human platelets: Energy metabolism in human platelets has been presumed to be associated primarily with aerobic glycolysis, although platelets contain abundant mitochondria and respire actively. In continued collaboration with J. Muenzer (formerly, NIAMDD; now at Case Western Reserve University), we have investigated the effects of known inhibitors of mitochondrial metabolism on isolated, intact human platelets. The endogenous rate of respiration was completely inhibited by rotenone, antimycin, cyanide and oligomycin. Significantly, the oligomycin block was relieved by uncouplers of oxidative phosphorylation. In contrast to the endogenous respiration, the thrombin-induced burst of oxygen consumption was only partially sensitive to these inhibitors. Inhibitors of platelet function (protaglandin $E_1$ and salicylates) strongly suppressed the thrombin-induced burst, but had minimal effects on the endogenous respiration. Thus, marked differences were observed in the action of inhibitors on the two types of respiration. Our data indicate that respiratory chain phosphorylation provides an important mechanism for energy conservation in human platelets.

Significance to Bio-medical Research and to the Program of the Institute:

This investigation, because of its fundamental nature in attempting to elucidate vital biochemical mechanisms common to all aerobic cells, has obvious importance for biochemical research in general. This is shown by the numerous collaborative projects in which we have participated within and without the NIH during the past years. This investigation also provides a basis for understanding bioenergetic problems associated with parasitism. Studies of energy metabolism in parasites, in addition to their intrinsic and comparative interest, contribute toward a more rational understanding of the host-parasite relationship, and may provide information for chemotherapeutic exploitation.

Proposed Course of the Project: The parasite studies will be continued with emphasis on the mechanisms of electron transport and energy conservation in cestodes. Biochemical studies on the aerobic metabolism of Entamoeba histolytica will be directed toward amino acid metabolism and delineation of the pathways involved in adenine and pyridine nucleotide biosynthesis. Our collaborative investigation of the electron transport chain and respiratory enzymes of Staphylococcus aureus will be continued.

The mammalian studies will be resumed with emphasis on the role of non-heme iron in oxidative phosphorylation. Studies of cardiac muscle mitochondria in vitro and in vivo will be undertaken with Dr. J. Dvorak and Prof. L. Ernster (University of Stockholm). Investigation of the energy metabolism in human platelets will be continued with an attempt to isolate functionally competent mitochondria.

methods, including polyacrylamide disc gel electronphoresis, that unlike the mammalian enzyme DT-diaphorase, there are two distinct dehydrogenase proteins in E. histolytica which are specific for each of the two reduced nucleotides Partial purification of the soluble enzymes has been accomplished by sonication, acetone precipitation and gel electrophoresis. NADPH dehydrogenase is approximately 20-times more active than NADH dehydrogenase. Inhibition observed in the presence of antimalarial drugs (atebrine, primaquine chloroquine) and chlorpromazine indicates that these enzymes are flavoproteins. Ferricyanide, 2,4-dichloroindophenol, and quiniones served as efficient electron acceptors. Typical activities of the diaphorases with menadione as electron acceptor were 85 nanoatoms O/min/mg protein for NADH and 1600 nanoatoms O/min/mg protein for NADPH.

3. Nucleotide metabolism in E. histolytica: In collaboration with Dr. T. Takeuchi (Visiting Fellow, Keio University, Japan), a study of adenine and pyridine nucleotide metabolism in E. histolytica was initiated this year. As a basis for this work, it was necessary first to determine the nucleotide content of these parasites. The following average values in nanomoles per mg protein were found: NAD 1.3, NADH 0.6, NADP, 1.0; NADPH 0.4, AMP 3.1, ADP 5.3 and ATP 5.3. Sonicated preparations of E. histolytica exhibited a Mg- and 2,4-dinitrophenol-activated ATPase which was inhibited by oligomycin. Although the activity of the ATPase is surprisingly low (10 nanomoles ATP hydrolyzed/min/mg protein), it is qualitatively similar to the mammalian enzyme(s). Only marginal activity was found for the $^{32}P_i$-ATP exchange reaction.

The most significant finding in this preliminary work was that E. histolytica trophozoites readily synthesized ATP from endogenous precursors at 30°. Synthesis of ATP occurred under aerobic or anaerobic conditions. Addition of ADP and $P_i$ greatly enhanced (500%) the formation of ATP. To a lessor extent, AMP and either $P_i$ or $PP_i$ also enhanced the biosynthesis of ATP. In contrast, storage of the intact trophozoites for 1-3 hrs at 4° results in a progressive loss of ATP. This observation is consistent with the marked morphological deterioration of amebae seen during the same time period.

4. Respiratory metabolism of Staphlococcus aureus: In collaboration with Dr. T. S. Theodore, LM, NIAID, an investigation of the respiratory enzymes of mesosomal and plasma membrane fractions of S. aureus was initiated late in the year. Preliminary findings disclosed that succinate, malate, lactate, glycerol-3-phosphate and NADH oxidases were most active in the plasma membrane fraction. Mesosomes showed only marginal oxidase activities. Currently, studies on the individual dehydrogenases are being done to determine if any increased levels of specific respiratory enzymes are associated with the mesosomes.

B. Mammalian Studies

1. Rat liver mitochondria: Most of the work with mammalian tissues this year was restricted to the use of rat liver mitochondria as a

Honors and Awards:

E. C. Weinbach was invited to the New Jersey Medical School to present two seminars on "The Aerobic Metabolism of Entamoeba histolytica" and "Bioenergetic Phenomena in Human Platelets", January 26-27, Newark.

Publications:

Weinbach, E.C. and Diamond, L.S.: Biochemistry of Entamoeba histolytica. I. Aerobic Metabolism. Exp. Parasitol. (In Press).

Kark, R.A.P., Weinbach E.C., and Engel, W.K.: Oxidative metabolism in small samples of normal and diseased muscle. Proceedings of the 2nd International Congress on Muscle Diseases, Perth, Australia, Excerta Medica (B. Kakulas, editor) 1971-1972, (In Press).

Serial No. NIAID 123-E
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biophysical Parasitology

Previous Serial Number: Same

Principal Investigator: James A. Dvorak

Other Investigators: Norman A. Luban

Cooperating Units: Biomedical Engineering and Instrumentation Branch, DRS and Television Engineering Section, CC

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 34/12 | | | 34/12 |
| Professional | 24/12 | | | 24/12 |
| Other | 10/12 | | | 10/12 |

Project Description:

Objectives: To develop and utilize biophysical techniques for the study of parasitological problems of medical importance. Although major emphasis is still being directed toward a better understanding of the host-parasite interaction resulting in Chagas' disease, the techniques developed with this program are also being utilized in a study of the penetration kinetics of red blood cells by malaria merozoites. The approach is two-fold: (1) to obtain a clearer understanding of the interaction at the cellular and subcellular level of the parasite with the host cell and (2) to relate this information to what is observed in a population of infected cells in vitro and, hopefully, the general course of events in vivo.

Methods Employed:

(1) In vitro cultivation

The controlled-environment culture system developed with this

program is used routinely to maintain in vitro steady-state physiologic conditions. This has permitted an analysis of the influence of numerous physical and/or chemical parameters on the overall host-parasite interaction. A modification of the basic system incorporating a chamber suitable for the analysis of non-adherent cells without a support membrane is also being utilized.

(2) Quantitative microscopy

Analytical techniques have been greatly improved and extended by the incroporation of a video integrator system and low light level devices. This equipment maximizes the effectiveness of several analytical microscopy techniques and permits the determination of the effects of physical and/or chemical parameters at a level of precision previously unobtainable with living biological systems. Furthermore, with the video format, data may be analyzed as it is generated or accumulated for analysis at a later time.

Major Findings:

(1) The interaction of Trypanosoma cruzi with vertebrate cells in vitro

The study of the interaction of T. cruzi with vertebrate cells has been extended to include increasingly more complex biological and/or physical parameters.

(a) A quantitative analysis of the effects of host cell type upon the ability of trypomastigotes to penetrate the cell revealed that skeletal muscle cells are penetrated by 22 times more parasites than kidney cells, and 4 times more parasites than HeLa cells under identical conditions. Although the mechanism responsible for these observations is not known, it is interesting to note that these observed differences correlate well with reported differences in the thickness of the cell coat of these vertebrate cells. Thus, the observed differences could represent the effectiveness of a physical barrier (the cell coat) to the penetration of the host cell by the parasite.

(b) Inhibitor studies have been initiated in an attempt to elucidate differences and/or similarities in major metabolic pathways between T. cruzi and vertebrate cells. The use of cytosine arabinoside, a potent inhibitor of DNA synthesis in vertebrate cells, indicated that intracellular parasites may possess a DNA synthetic pathway which is insensitive to the action of the drug or a DNA synthetase step which is effected to a lesser degree than vertebrate cells.

(c) The intracellular cycle of two strains of T. cruzi was observed using trypomastigotes obtained from insects and trypomastigotes obtained from cell cultures. The lag period prior to the onset of reproduction was statistically the same as that reported previously. The doubling times of parasites derived from insects was 7.5-8.6 hrs. However, the doubling times were not static. During cell culture passage of these parasites, their doubling times

lengthened to 11.3-11.5 hrs. About 7-10 weeks were required for the doubling times to stablize. Parasite reproduction proceeded for about 9 generations. The net result is the completion of the intracellular cycle in 4.5 days with parasites derived from insects vs 6.5 days following stabilization of the parasite's doubling time in cell culture. There are marked changes in physical-chemical parameters in the transition of parasites from the environment of the insect gut to the vertebrate cell. These changes, in turn, apparently influence physiologic parameters which govern certain aspects of the intracellular cycle of the parasite. The ability to demonstrate a change in the life cycle of T. cruzi during the transition from the invertebrate host to the vertebrate host could aid in our understanding of the course of the disease in vivo.

(d) One physical parameter which significantly affects the intracellular cycle of T. cruzi is temperature. As temperature is increased from 29°-38°C, the lag period prior to the onset of reproduction decreases. However, as temperature increases from 32°-38°C the reproduction time increases linearly, whereas at 29°C the reproduction time also lengthens. The net effect on the intracellular cycle between 32° and 38°C is essentially zero; at 29°C the cycle lengthens to 9 days. Thus, within limits, the intracellular cycle of T. cruzi could be considered a biological clock. Furthermore, the lag period prior to parasite reproduction and the reproductive phase consist, physiologically, of two distinct but interrelated processes. The metabolic pathways and/or enzyme systems required in the reproductive phase of the parasite are both temperature sensitive and thermolabile in a manner different from what occurs in normal vertebrate cells. These data could offer an avenue for subsequent biochemical analysis of specific events in the intracellular cycle.

(e) Fully differentiated and functional myocardial cells are being used as an in vitro model for Chagasic myocarditis. In "beating" myocardial cells, neither the frequency nor the amplitude of the beat changes during the penetration of the cell by the parasite. Beating myocardial cells also continue to beat during the reproductive phase of the parasite within the cell. Prior to differentiation of the parasite, however, the host cell stops beating. If an infected cell is beating in synchrony with uninfected cells, the uninfected cells continue to beat normally.

(2) Instrumental methods development

(a) An analytical system utilizing a video format has been developed. The video format provides a convenient display in which density, geometry and time are all precisely interrelated. If the desired analytical procedure can be performed directly, control is passed from the operator to an integrator equipped with logic circuitry and analysis begins. However, if analysis can not proceed due to difficulties in pattern recognition, for example, control is returned to the operator who manually sets externally generated boundary conditions. Thus, the vast majority of problems inherit to video analytical systems are bypassed with one simple, inexpensive maneuver.

(b) A method of interference reflection microscopy is being used for an analysis of events occurring at the interface between a vertebrate cell and its environment at a z-axis resolution of at least 50 Å. The technique is being used to study the actual penetration of a vertebrate cell membrane by a trypomastigote. Preliminary data indicate that a fiberoptically-coupled, second generation microchannel plate can act as a suitable detector at light levels which are not injurious to the biological objects being studied.

(3) Mathematical modeling of host-parasite interactions.

The in vitro parameters analyzed to date have been used to construct a mathematical model of the course of a T. cruzi infection in vivo. The model is now sufficiently complete to allow the generation of a hypothetical parasitemia curve which correlates well with actual data reported from animals which pass through an acute phase of the disease followed by an immune response. Although all parameters analyzed to date must be considered in the generation of a mathematical model, some parameters are considerably more important than others. This realization has greatly aided our understanding of the overall host-parasite interaction.

(4) Malaria studies

This laboratory has been collaborating with Dr. L. H. Miller in a study of the interaction of vertebrate red blood cells with malaria merozoites. Work has centered upon an analysis of the effects of modification of the surface of the red cell and host cell range upon the host-parasite interaction. See NIAID 132 for details.

Significance to Biomedical Research and the Program of the Institute:

(1) The study of host-parasite interactions at the cellular and subcellular level is a topic of critical importance. The outcome of this interaction directly influences the physiologic state and survival potential of both the host and the parasite.

A clearer understanding of the entire interaction has signigicantly improved the probability of finding biological "pressure points" for attempted interdiction of the parasite's cycle. For example, interdiction of the penetration phase of the interaction will totally interupt the natural life cycle of the parasite. Mathematical analysis of the interaction indicates that a humoral immune response is one method by which this could be accomplished. The effects of pharmacologically active compounds upon several aspects of host-parasite interactions is another approach which it is now feasible to pursue.

The general applicability of this research approach to the study of other human infectious diseases has been demonstrated by our ability to utilize our techniques in the analysis of the interaction of several protozoan parasites with vertebrate cells. The wealth of comparative information which is being accumulated should be of considerable value in the understanding of parasitic diseases in general.

(2) The methodology and research philosophy being developed are directly applicable to other areas of biomedical research. For example, the controlled-environment culture system developed with this program is now in routine use in laboratories with such diverse interests as oncology, immunology, neurophysiology and morphogenesis. The video integrator system developed with this program is being used for analysis of macroscopic as well as microscopic images in basic research and diagnostic clinical-medical fields.

Proposed Course of the Project: Research will continue in an attempt to further our understanding of the interaction of T. cruzi with vertebrate cells in vitro and relate this information to the course of Chagas' disease in nature. Basic information obtained with the controlled-environment culture system and video integrator will be utilized in more complex in vitro model systems. Verification of the methematical model will require analysis of immunologic parameters in the interaction.

The basic methodology and research philosophy developed with this program will be used for the analysis of other host-parasite interactions.

Honors and Awards:

An invited lecture entitled: "The development of the Biophysical Parasitology Program at the NIAID with special emphasis on its applicability to biomedical research in general" was presented by J. A. Dvorak to the Board of Scientific Directors, NIH on Nov. 1, 1972.

An invited lecture entitled: "A new approach to the study of human parasitic diseases via quantitative light microscopy with notes on its applicability to other research areas" was presented by J. A. Dvorak at the Armed Forces Institute of Pathology on Jan. 16, 1973.

The opening address to the Symposium: Dialogues in Microscopy entitled "The man-machine interface in microscopy" was presented by J. A. Dvorak in New York City on May 14, 1973.

An invited paper entitled: "Low light level microscopy in Biology-problems and promises" was presented by J. A. Dvorak at the Symposium: Dialogues in Microscopy in New York City on May 15, 1973.

J. A. Dvorak was an invited participant in the Symposium on Trypanosomiasis and Leishmaniasis at the Walter Reed Army Institute of Research on April 12-13, 1973.

Invention: A controlled-environment culture chamber for high resolution light microscopy. U. S. Patent applied for, Feb. 1971. All claims allowed by U. S. Dept. of Commerce Patent Office, Oct. 12, 1972. U. S. Serial No. 233-194.

Publications:

Dvorak, J. A., Clem, T. R., and Stotler, W. F.: The design and construction of a computer-compatible system to measure and record optical retardation with a polarizing or interference microscope. J. of Microscopy 96: 109-114, 1972.

Dvorak, J. A., and Schmunis, G.: Trypanosoma cruzi: Interaction with mouse peritoneal macrophages. Exp. Parasitol. 32: 289-300, 1972.

Miller, L. H. and Dvorak, J. A.: Visualization of red cell membranes of lysed malaria infected cells by differential interference microscopy. J. Parasit. 59: 202, 1973.

Dvorak, J. A. and Hyde, T. P.: The interaction of Trypanosoma cruzi with vertebrate cells in vitro. I. Individual host cell-parasite interactions at the cellular and subcellular level. Exp. Parasit. (In press)

Hyde, T. P. and Dvorak, J. A.: The interaction of Trypanosoma cruzi with vertebrate cells in vitro. II. Quantitative analysis of the penetration phase. Exp. Parasit. (In press).

Serial No. NIAID 123-F
1. Parasitic Diseases
2. Physiology & Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Terminal Electron Transport in Parasites

Previous Serial Number: None

Principal Investigator: Dan R. Harlow

Other Investigators: Eugene C. Weinbach, Louis S. Diamond, Guillermo Pacheco and R. Frederic Knauft

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 5/12 | | | 5/12 |
| Professional | 5/12 | | | 5/12 |
| Other | | | | |

Project Description:

Objectives: To elucidate the fundamental biochemical mechanisms of terminal electron transport by which parasites oxidize reduced compounds under both aerobic and anaerobic conditions.

Methods Employed:

(1) Cestode mitochondria. Mitochondria are isolated from adult Hymenolepis diminuta. The life cycle is maintained in the laboratory using Tenebrio molitor and Tribolium spp. as intermediate hosts and Sprague-Dawley rats as definitive hosts. Adult tapeworms are harvested at 17 days post-infection by flushing from the intestine and washing them clean of intestinal contents with buffered Krebs-Ringer solution. The harvested tapeworms are homogenized and the mitochondria isolated by differential and sucrose density gradient centrifugation. The mitochondria obtained are much "cleaner" than heretofore possible starting with a whole helminth homogenate. The isolated mitochondria are examined by low-temperature spectroscopy for presence of cytochromes and cytochrome-like compounds.

(2) Amoeba transhydrogenase. Axenically-grown Entamoeba histolytica are washed in buffered saline or buffered sucrose (L. S. Diamond) and examined for enzymatic activity either as intact organisms or sonicated organisms.

(3) Filariid oxidative metabolism. Microfilariae of Dirofilaria immitis harvested from the blood of infected dogs (Pacheco and Knauft) are incubated in the polarograph (Clark oxygen electrode) and the oxygen removal is measured. Substrate and specific inhibitor effects on oxygen removal are noted.

Major Findings:

(1) Cestode mitochondria: Since the arrival of the principal investigator at NIH during August of 1972, he has developed the H. diminuta life cycle to a point capable of producing the large numbers of worms (50 g per assay) necessary for this study. Soon after his arrival, the equipment required for mitochondrial isolation was ordered and it was received after the first of the year. Test procedures with the new equipment compared favorably with previous experience obtained during the design of this isolation technique. The life cycle production and equipment are now ready for a vigorous pursuance of the problem.

(2) Amoeba transhydrogenase. The enzyme "transhydrogenase" which functions in electron transport between NAD and NADP was found in E. histolytica. This enzyme has not been previously reported from any protozoan species, either free-living or parasitic. Transhydrogenase is of particular interest in E. histolytica since a diaphorase activity has been found (E. C. Weinbach) which exhibits a much higher activity with NADPH than with NADH. Since the glycolytic sequence is operative in E. histolytica, transhydrogenase may function as a means of oxidizing NADH reduced during glycolysis via NADPH and the diaphorase reaction mentioned above.

The transhydrogenase of E. histolytica resembles Kaplan's transhydrogenase from mammalian sources in that it favors the reaction NADPH⟶NAD over NADH⟶NADP in vitro, although in a ratio of 3:1 rather than 10:1 as Kaplan reported. Using acetylpyridine-deamino NAD (AC-D-NAD), the activity of the enzyme is 3.6 nanomoles AC-D-NAD reduced/min/mg protein. The value for rat liver mitochondria is 4.1 nanomoles AC-D-NAD reduced/min/mg protein, a comparable value. Storage for 24 hours @ 4°C increased the activity of the amoebal enzyme to about 9.0 nanomoles AC-D-NAD reduced/min/mg protein while sonication increased the activity to a lesser degree. The reverse reaction, NADH⟶NADP, measured using acetylpyridine NADP (AC-NADP) is 1.28 nanomoles/min/mg protein in intact amoebae or about 1/3 the activity observed for NADPH⟶NAD.

(3) Filariid oxidative metabolism. Studies were initiated in collaboration with Drs. Knauft and Pacheco on oxidative metabolism in D. immitis microfilariae. These parasites had an endogenous oxygen utilization of 24 nanoatoms oxygen/min/mg protein, which was increased to 35

nanoatoms oxygen/min/mg protein after addition of 15 mM glucose. Both the endogenous and glucose-supported oxygen uptake were totally inhibited by KCN (1 mM), antimycin (10 μg/ml), and rotenone (10 μM); but only partially inhibited by Amytal (1 mM) and malonate (10 mM). Sonication for 1 minute abolished oxidative activity.

The complete sensitivity of D. immitis microfilariae to typical inhibitors of aerobic metabolism sets it apart from other parasites and suggests similarities to mammalian oxidative mechanisms.

Significance to Bio-medical Research and the Program of the Institute:

The body of evidence accumulated on parasite biochemistry indicates primarily anaerobic metabolism. This is confused by the general observation that these parasites will utilize oxygen when present. The presence or absence of mammalian-type cytochromes is not yet satisfactorily established. Currently, the terminal electron transport mechanisms of parasites are not known - thus, whether they are similar to or different from the mammalian host's systems also is not known. It is of primary importance to determine these mechanisms both from the aspect of possible chemotherapeutic value and the aspect of intrinsic scientific merit.

Proposed Course of the Project: The cestode mitochondria work will proceed toward a definitive characterization of mitochondrial cytochromes. This work will be the first definitive study of cytochromes on a "clean" mitochondrial preparation from a parasitic helminth.

The transhydrogenase study with E. histolytica is nearing the completion and publication phase.

Studies on terminal electron transport of the filariid nematode, D. immitis, will be amplified. From the results of our initial study, it appears that interesting mechanisms of terminal electron transport perhaps similar to mechanisms found in mammals may occur in this parasite.

Serial No. NIAID 125-A
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Toxoplasmosis

Previous Serial Number: Same

Principle Investigator: Harley G. Sheffield

Other Investigators: Marjorie L. Melton

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 21/12 | | | 21/12 |
| Professional | 21/12 | | | 21/12 |
| Other | | | | |

Project Description:

Objectives: The study of Toxoplasma gondii infections in cats to compare with the course of the disease in man; to determine the stages and sites of development of the parasite in the cat; to investigate the possibility of other animals serving as natural hosts; to determine possible mechanisms by which oocyst infections can be acquired by man and animals.

Methods Employed: Cats are infected by feeding them cysts in mouse brain or oocysts isolated from feces of infected cats. Oocysts are collected by zinc sulfate or sucrose flotation. Infectivity of concentrated oocysts is determined by feeding them to mice and subsequent checking for organisms or anti-toxoplasma antibodies. Tissues from infected animals are used for histological or ultrastructural studies. Cultured cells are used for in vitro studies of development of sporozoites which have been artificially excysted from oocysts and inoculated into cultures.

Major Findings: Studies to determine the refractoriness of cats to reinfection with Toxoplasma gondii have been continued. Generally cats having anti-toxoplasma antibody are refractory to reinfection for several months or more after the initial infection. Since the RH strain does not produce oocysts in cats it has been used in efforts to produce immunity. Cats were infected with cysts of RH strain T. gondii and developed low dye test titers but did not pass oocysts. Challenge with cysts of C-56 strain resulted in normal oocyst production and rapid elevation of antibody titers. The etiology of this response is being investigated.

Preliminary data indicated last year that intramuscular treatment of infected cats with pyrimethamine and sulfadiazine resulted in a delay in oocyst production. Further studies have verified those results. Oral administration of the drugs also has been shown to delay oocyst output to a similar degree. Studies are in progress to determine the stage of infection at which the drug is active. It has not been determined whether the organisms invade the extraintestinal tissues and little antibody to toxoplasma was found by dye test one month after infection.

Earlier studies indicated that pyrimethamine effectively inhibits multiplication of T. gondii in cultured cells within 24 hours after infection. Recent work has shown, by light microscopy, that the parasites are morphologically distorted after 12 hours incubation with the drug. Electron microscope studies are planned to further elucidate this finding. The effectiveness of U-21, a lincomycin derivative, has been tested against T. gondii in cultured cells. No effect on multiplication was observed by light microscopy after 24 hours but the drug may have some effect in 48-72 hours as indicated by reduced numbers of organisms in the cells.

Oocysts resembling those of T. gondii but probably related to Sarocystis muris have been obtained from Dr. Gordon Wallace (see 67-D). Passage through mice and cats has provided material for study of the parasite in cultured cells. Artificially excysted sporozoites were inoculated into monkey kidney cells and observed for 7 days. Sporozoites invaded and multiplied like those of T. gondii. Electron microscope studies are planned.

In conjunction with others studying drug effect against chronic toxoplasmosis in mice (see 132-C) the presence of organisms in eyes of chronically infected mice was investigated. Parasites were isolated from eyes of 7 of 10 mice infected with C-37 strain T. gondii. Eyes of 46 mice on drug test (including controls) were subinoculated and produced infection in all but 6 cases indicating a high prevalence of infection in eyes of chronically infected mice. Differentiation of cysts or proliferative forms is now being done. (Drug study results are reported in 132-C.)

Significance to Biomedical Research and the Program of the Institute:

The identification of the cat as the natural host for T. gondii provides a new insight into the epidemiology of toxoplasmosis and could explain the wide-spread existence of anti-toxoplasma antibodies in man and animals.

In addition, the linking of T. gondii, whose classification has been uncertain for many years, to the coccidia provides the investigator with many model systems which could be used for comparison purposes in the study of toxoplasmosis in normal and abnormal hosts. The cat's role as a reservoir animal may be significant in the cosmopolitan distribution of the disease. Information on the immunological aspects of the disease in cats would show the possibility of reinfection and may provide new leads relative to the cat being an important reservoir of the organism. Further elucidation of the various stages of the life cycle in the cat would contribute to a better understanding of the disease in man. Basic information on the biology of the organism and relationship to other protozoa could give some explanation for the parasite's unusual lack of host specificity and unique means of multiplication.

Proposed Course of Project: Cultivation of T. gondii in vitro will be continued. Attempts to determine the factors which allow the parasite to divide by schizogony in the natural host but by endodyogeny in abnormal sites will be made. The immunological aspects of the disease in the cat will be examined to supplement existing data on the importance of cats as reservoir hosts. Studies on related organisms will be continued for comparative purposes and to examine the possibility that other coccidia would be infective for man.

Honors and Awards:

Awarded Public Health Service Commendation Medal.

Invited lecturer on biology of Toxoplasma gondii, National Institutes of Health Graduate School.

Editor, Proceedings of the Helminthological Society of Washington

Consultant, Journal of Parasitology

Member, Editorial Board, Journal of Protozoology

Chairman, Placement Committee, American Society of Parasitologists.

Publications:

Fayer, R., Melton, M.L., and Sheffield, H.G.: Quinine inhibition of host cell penetration by Toxoplasma gondii, Besnoitia jellisoni, and Sarcocystis sp. in vitro. J. Parasit. 58: 595-599, 1973.

Serial No. NIAID 125-B
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: In-vitro cultivation of Coccidia

Previous Serial Number: None

Principal Investigator: Franklin A. Neva

Other Investigators: Gordon D. Wallace, Pacific Research Section and Harley G. Sheffield

Cooperating Units: University of Illinois School of Veterinary Medicine

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 13/12 | | | 13/12 |
| Professional | 1/12 | | | 1/12 |
| Other | 12/12 | | | 12/12 |

Project Description:

Objectives: Since Toxoplasma gondii has recently been found to be a coccidian type parasite it seems appropriate to consider the potential of other coccidia as human pathogens.

Assessment of the pathogenicity of other coccidia for man is particularly needed since practical serologic tests to indicate infection with these organisms are virtually non-existant. Therefore, if various coccidia could be easily cultivated in-vitro such a system might provide antigens for serologic tests or a system for assay of antibody against the organism. In addition, basic information about the biologic behavior of these organisms might be gained by their study under conditions of in-vitro cultivation in cell cultures.

Methods Employed: Standard methods of cell culture with primary as well as cell-line cultures are used. Inoculated cultures are observed directly for evidences of parasite growth and cultures or material from harvested fluids are fixed and stained. Oocysts of Isospora canis were kindly provided by Dr. Kenneth Todd of the University of Illinois. Dr. Gordon Wallace of Pacific Research Section provided oocysts of the murine Sarcocystis strain WC1170. Oocysts are permitted to sporulate and excysted with the same techniques as has been used for Toxoplasma. Animals are inoculated and tissues are obtained for histologic study by standard techniques.

Major Findings: 1. Several different types of cultures inoculated with excysted sporozoites of I. canis were observed for slightly over 8 months. Although abortive replication was believed to have occurred in some of the cultures, continued growth was not obtained. However, apparently viable and motile sporozoites persisted within cells for over 8 months. An experiment designed to test proof of infectivity of cultured sporozoites in puppies was not conclusive.

2. The other organism studied in cell cultures is one that has an oocyst stage indistinguishable from the oocyst of Toxoplasma, but after inoculation into mice it produces Sarcocysts and is therefore believed to be Sarcocystis muris. Sporozoites of this organism were found to multiply in a limited fashion, like the trophozoites of Toxoplasma. However, after about 6 weeks in cell cultures the few infected cyst-like cells began to diminish and the organisms apparently stop multiplying. Mice have been inoculated with similar material but have not yet been examined. See Project Report No. 67- for more information on the biology and nature of this organism.

Significance to Bio-medical Research and the Program of the Institute:

One of the requirements for a serologic test that will detect evidence of infection with a microorganism is the ability to grow the agent for preparation of suitable antigens. Until this is achieved it will not be possible to test human sera for example for evidences of infection with these organisms. In addition, it will be important to determine extent of cross-reactions, if any, of the other coccidia with Toxoplasma.

The long experience of some of the Staff of the Laboratory of Parasitic Diseases with Toxoplasma gondii will permit a well-balanced evaluation and comparison of the Sarcocystis-type organism with other coccidia.

Proposed Course of the Project: Further attempts will be made to cultivate the Sarcocystis organism in cell-cultures. Inoculation of cultures with sarcocysts from infected muscle of the mouse are planned. It is likely that other members of the Laboratory of Parasitic Diseases' Staff will research interests more directly related to toxoplasmosis will take over certain aspects of the work with this organism.

Serial No. NIAID 125-I
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Electron Microscope Studies on Parasites

Previous Serial Number: Same

Principle Investigator: Harley G. Sheffield

Other Investigators: None

Cooperating Units: Animal Parasitology Institute, ARS-USDA, Beltsville, Maryland

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 5/12 | | | 5/12 |
| Professional | 3/12 | | | 3/12 |
| Other | 2/12 | | | 2/12 |

Project Description:

Objectives: To determine the fine structure of various parasites in relation to their taxonomy, life cycles, reproduction and other physiological functions.

Methods Employed: Parasites are obtained from their host animals or culture at the developmental stage desired or after appropriate experimental procedures. The parasite tissue is processed, sectioned and then studied with the electron microscope and/or cytochemical methods.

Major Findings: Lack of technical assistance forced suspension of a portion of this project. Much of the time reported above has been utilized in the training of a new assistant.

The development of the intestinal tract of Dirofilaria immitis larvae in the Malpighian tubules of Anopheles quadrimaculatus mosquitoes was studied. Examination of larvae as early as 3 days after infection of the mosquito

showed the intestine to be a column of cells, usually 6-8 visible in cross sections, surrounding a minute lumen. Numerous vesicles and associated membranes in the apical area of the cell suggest that material may be emptied into the lumen forcing it to expand. Prominent septate junctional complexes connect the lateral borders of the cells at their apical ends. The small lumen, absence of recognizable food material and presumed lack of connection with the anal vesicle and pore suggest that the intestine is not functional at this stage. After the first moult (6 days) the lumen enlarges. At the 2nd moult (9 days) the material filling the anal vesicle and forming the anal plug is lost and the intestine becomes patent. At the same time material resembling that seen in the area surrounding the larva is found in the lumen.

Studies in collaboration with Dr. Ronald Fayer, Animal Parasitology Institute, ARS-USDA, Beltsville, Md. have been initiated to examine the fine structure of the infective stages of Sarcocystis fusiformis, a toxoplasma-like parasite of cattle. The infective forms develop in the intestinal wall of dogs fed beef hearts containing cysts of the parasite. Intestinal tissue from infected dogs is in the process of being examined. Mature oocysts passed from similarly infected dogs have been excysted and the sporozoites studied by electron microscope. Preliminary observations indicate that they have most of the characteristics seen in other coccidian sporozoites. Further investigation should aid in understanding the relationship between Toxoplasma gondii, various Sarcocystis spp. and known Isospora spp.

Significance to Biomedical Research and the Program of the Institute:

Continued investigation of the similarities between Toxoplasma gondii and other coccidia provides new leads to the study of the biology and epidemiology of the former. Study of the differences of development in the natural host compared to that in man and other animals could provide insight into the mechanism ofhost specificity in this parasite. Study of related organisms will provide additional information which could allow a better understanding of the structure of T. gondii and its relation to the functioning of the organism.

Proposed Course of the Project: Studies on the endogenous stages of T. gondii in cat intestine under normal and experimental conditions will be continued. Development will also be studied in vitro as techniques permit. Mechanisms of penetration will be studied and supplemented with information obtained using other parasites. Other toxoplasma-like organisms will be studied as material is available and compared with existing data. Development of various aspects of Dirofilaria immitis larvae will be studied with special emphasis on the digestive and excretory systems. A portion of the time will be used to provide other investigators with electron microscopic assistance when their needs arise.

Serial No. NIAID 125-K
1. Parasitic Diseases
2. Parasite Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Amebiasis studies in India

Previous Serial Number: Same

Principal Investigator: Louis S. Diamond

Other Investigators: Milford N. Lunde

Cooperating Units: Dr. L. N. Mohapatra, All-India Institute of Medical Sciences, New Delhi, India (PL 480 Project Agreement No. 01066-1).

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 2/12 | | | 2/12 |
| Professional | 2/12 | | | 2/12 |
| Other | | | | |

Project Description:

Objectives: To evaluate an intradermal test for amebiasis using an antigen (Histolyticin) prepared from axenically cultivated Entamoeba histolytica in both a hospital and field situation; to determine the usefulness of the indirect hemagglutination test in assessing the effectiveness of drug therapy in amebic disease; to obtain data on the epidemiological significance of E. histolytica-like Laredo amebae.

Methods Employed: Clinical and parasitological evaluations using classical techniques. Serological tests using intradermal inoculation indirect hemagglutination, agar-gel, counter current immunoelectrophoresis. Stool, liver and pleural exudate culture.

Major Findings: To-date, a total of 456 individuals have been skin tested with Histolyticin antigen injected intradermally in doses varying from 1 to 16 μg amebic protein. Included in this group were 201 patients clinically and parasitologically assessed to be suffering from amebic disease, 191 patients with non-amebic parasitic disorders and 64 healthy individuals. No adverse reactions were observed in any of the individuals tested. Briefly, the results obtained with the skin test together with results of the IHA test (> 1:162) performed on sera obtained at the time of intradermal testing were as follows: Liver abscess - 33 cases of which 75.7% were positive by the ID test and 81.8% by the IHA test; amebic hepatitis - 37 cases, 35.1%, ID, 29.7% IHA; acute amebic dysentery - 28 cases, 89.3% ID, 85.7% IHA; non-dysenteric amebic colitis - 103 cases, 19.4% ID, 20.4% IHA; non-amebic parasitic diseases - 68 cases, 13.2% ID, 14.7% IHA: irritable bowel syndrome - 123 cases, 41.1% ID, 38.2% IHA; healthy controls - 64 cases, 3.1% ID, 14.1% IHA.

As a result of studies conducted at the Clinical Center (Kirkpatrick, Lunde and Diamond) with human volunteers and analysis of the data obtained in India a dose of 6 μg amebic protein is recommended for the ID test performed with Histolyticin antigen. Such a dose is below that known to induce sentization to the antigen and tends to produce fewer false positive reactions.

In an attempt to assess the usefulness of the IHA test in evaluating effectiveness of chemotherapy in amebic disease a follow-up study of IHA titers after successful treatment with metronidazole was performed on a limited number of liver abscess and non-dysenteric amebic colitis cases. In general in the cases of liver abscess there was an initial 6 to 9 fold decrease in titer within 5 to 9 months after treatment, followed by a period of constant titer for 2 to 3 months, then another comparable drop in titer over another period of 5 to 9 months. Moreover, persistence of antibody was dependent on the original level of antibody. In 9 cases with original titers ranging from 1:118,098 to 1:39,366 titers of 1:162 or more persisted for 17 months. In 7 of these cases which could be followed to 19 months the titers of all but one were still 1:162 or higher. In a 10th case with an initial titer of 1:1458 a titer of 1:162 was present at 15 months but was 1:54 at 17 months.

In 5 cases of non-dysenteric colitis with initial titers ranging from 1:1458 to 1:486, titers of 1:162 or more persisted for 3 months after treatment, then declined to 1:54 or less during the succeeding 8 months.

Significance to Bio-medical Research and the Program of the Institute:

The data obtained with the intradermal test indicates a usefulness of the Histolyticin skin test in a clinical setting, especially for the difficult to diagnose extra-intestinal forms of amebic disease.

Proposed Course of the Project: PL-480 Agreement No. 01066-1 is due to expire June 30, 1973. Since, there is still sufficient unexpended funds to carry the project through for another year it is anticipated that the Principal Investigator will request a year's extension without additional funds in order to provide an opportunity for the staff to bring all present research to a successful close, conclude analysis of the data and prepare material for publication.

Honors and Awards:

Invited participant, Workshop on Amebiasis, sponsored by the All-India Institute of Medical Sciences, New Delhi, December 1 and 2, 1972. Delivered the first Annual Om Prakash Memorial Lecture, December 2. Guest lecturer Madras Medical College, Madras, Grant Medical College and the Haffkine Institute, Bombay.

Serial No. NIAID 125-P
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunological studies on toxoplasmosis and other parasitic diseases

Previous Serial Number: Same

Principal Investigator: Milford N. Lunde

Other Investigators: Louis S. Diamond, Kendall G. Powers and William E. Collins

Cooperating Units: Primate Malaria Unit, Chamblee, Georgia

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 19/12 | | | 19/12 |
| Professional | 14/12 | | | 14/12 |
| Other | 5/12 | | | 5/12 |

Project Description:

Objectives: To differentiate and characterize the immune response in acute, chronic and reactivated latent toxoplasmosis and to evaluate diagnostic methods for detecting reactivation of the disease; to evaluate the skin test and other serological procedures as aids in the diagnosis of amebiasis; to evaluate and improve the indirect hemagglutination test for malaria.

Methods Employed: The dye, hemagglutination (IHA), complement-fixation, agar-gel diffusion, indirect fluorescent antibody (IFAT) tests are employed to determine serum antibody. Isolation of organisms is accomplished by inoculation of blood and other tissues into mice. Purified E. histolytica antibody is obtained by adsorption on sensitized bentonite and subsequent elution of antibody with glycine - HCl.

Sera are obtained from: a) Clinical Center, NIH, b) PL-480 project 01066-1, New Delhi, c) Primate Malaria Unit.

Major Findings:

1. Anti-human IgG and anti-rabbit IgG gamma globulins labeled with horseradish peroxoidase (HRP) have been used in the development of an indirect horseradish peroxidase (IHRP) test for the detection of antibody to Toxoplasma. Preliminary work indicates that these labeled antibodies may be used as a substitute for fluorescein in the serology of toxoplasmosis. Such a test can be performed with a light microscope thus eliminating the necessity of fluorescence equipment. Another advantage of HRP antibody is that it can be visualized with the electron microscope.

2. Sera from pregnant women with positive Toxoplasma IHA titers were obtained from the University of Alabama Medical Center and examined for 19 S antibody to Toxoplasma. Sufficient reliable data has not been obtained from these sera to determine the significance of Toxoplasma specific IgM antibodies at low levels, $\leq$ 1:40, during pregnancy.

3. An evaluation of the IHA test for amebiasis using antigen prepared from E. histolytica strain HK-9 indicates that titers less than 1:162 may be diagnostically insignificant. Results of the amebiasis skin test from the PL-480 project in India show that at a dose levelof 6$\mu$g protein (0.1 ml) non-amebic controls gave 10% false positive reactions whereas individuals with amebic liver disease and dysentery gave 20% false negative reactions. This data suggests that the skin test for amebiasis as currently employed is only useful under some circumstances in clinical diagnosis and of doubtful value in epidemiological studies to determine the incidence of invasive amebiasis in an endemic area.

4. Sera from malarial infections with P. falciparum do not react as well in the IHA test as do sera from P. vivax individuals. P. knowlesi antigen was used to sensitize erythrocytes. Hemagglutination antibody titers obtained after initial parasitemia tend to decline rather rapidly and respond with a rapid secondary rise following relapse.

5. Earlier work had shown that chlorinated lincomycin analogues are effective in treating acute toxoplasmosis in mice. Further studies that were carried out to assess their effectiveness against the cyst stage of the parasite demonstrated that the drugs did not effect cures in mice chronically infected with strain C-37. Some curative effect, however, was noted in mice chronically infected with the RH strain. No Toxoplasma was isolated from eight of 14 mice chronically infected with the RH strain and treated with chlorinated lincomycin analogues whereas Toxoplasma was isolated from six out of seven untreated chronically infected controls (see NIAID 132-C).

In addition to the above, routine IHA and/or dye tests are performed to provide serological data for use in other experimental work in this laboratory (see also NIAID 125-A).

Significance to Bio-medical Research and the Program of the Institute:

The search for improved sero-diagnostic tests in parasitic diseases and a better understanding of how these relate to the clinical situation and the experimental host in the laboratory will be of value not only to the clinician but also to the investigator seeking a better understanding of host parasite relationship and the immune factors involved.

Since it has been shown that the primate malaria antigen, P. knowlesi is not as sensitive as the IFAT in human malaria, continued studies toward the improvement of sensitivity of the malaria IHA test will lead to reliable test available for use in the sero-diagnosis and sero-epidemiology of malaria.

The need for continued studies of drugs with anti-toxoplasma activity is warranted because of the hematological side effects of current pyrimethamine-sulfadiazine therapy. Treatment of toxoplasmosis during pregnancy with pyrimethamine and sulfadiazine is contraindicated because of toxicity.

Proposed Course of Project: The demonstration of an IHRP antibody titer to toxoplasmosis comparing with the IHA titer suggests that once the system has been refined and evaluated it may be useful in other parasitic diseases such as amebiasis, malaria, Chagas', etc.

A major effort will thus be directed to further the development and evaluation of horseradish peroxidase labeled antibodies and antigens for use in clinical and experimental parasitology. The sero-diagnosis of malaria especially with the IHA will continue to be evaluated in cooperation with the primate Malaria Unit, NIAID.

Since work now in progress and recently completed indicates that the chlorinated lincomycin analogues are effective in acute murine toxoplasmosis and of doubtful curative effect against the cyst stage of T. gondii; it is expected that studies with these drugs will be terminated. If other compounds become available that merit study for anti-toxoplasmosis actively they will be evaluated.

Additional sera from PL-480 project 01066-1 in India will be examined for anti-E. histolytica antibody.

Honors and Awards:

Invited to present a paper on the laboratory diagnosis of toxoplasmosis at the Public Health Laboratory Directors Conference, The American Public Health Association, in Atlantic City, November 12, 1972.

Invited to present a paper on the serological diagnosis of amebiasis at a workshop on amebiasis, sponsored by the All-India Institute of Medical Sciences, New Delhi, December 1972.

Invited lecturer on the serological and immunological aspects of Toxoplasma gondii, NIH Graduate School.

Publications:

McMaster, P. R. B., Powers, K. G., Finerty, J. F., and Lunde, M. N.: The effect of two chlorinated, lincomycin analogues against acute toxoplasmosis in mice. Am. J. Trop. Med. Hyg. 22: 14-17, 1973.

Lunde, M. N.: Laboratory methods in diagnosis of toxoplasmosis. Health Lab. Sci. (In Press), 1973.

Serial No. NIAID 132
1. Parasitic Diseases
2. Malaria
   a. Primate Malaria
   b. Human Malaria
3. Bethesda, Maryland and Chamblee-Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Administration and Planning of Section

Previous Serial Number: Same

Principal Investigator: Louis H. Miller

Other Investigators: William E. Collins and Richard H. Glew

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 29/12 | | | 29/12 |
| Professional | 5/12 | | | 5/12 |
| Other | 24/12 | | | 24/12 |

Project Description:

Support and organization for the Section is directed through the Section and Unit Heads in close cooperation with the Laboratory Chief. Dr. Miller is responsible for and supervises patient care at the Human Volunteer Unit at the Atlanta prison. Supervision over personnel and maintenance of the Unit on Primate Malaria at Chamblee, Georgia, is handled by Dr. Collins.

Dr. Richard Glew became Medical Officer in Charge of the Unit on Human Malaria upon the completion of service by Dr. William A. Howard. Dr. W. A. Krotoski left the Unit on Primate Malaria to complete his training in preventive medicine. Dr. David Wyler joined the Unit on Human Malaria as Medical Officer. Mr. Raymond Hale, Head MTA, transferred to the Staff of the Atlanta Federal Prison.

Cooperative studies with scientists outside the NIH included Dr. Peter G. Contacos, Center for Disease Control, Drs. McWilson Warren and Geoffrey Jeffery, Central America Malaria Research Station, El Salvador, Professor Anthony Epstein, Department of Pathology, Bristol, England, Professor P. C. C. Garnham, Imperial College Field Station, Berkshire, England, Dr. Ruth Nussenzweig, New York University, Dr. Masamichi Aikawa, Case Western Reserve Medical School, Cleveland, and Dr. Thomas M. Sodeman, University of Michigan.

Serial No. NIAID 132-A
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mechanism of Invasion of Malarial Merozoites

Previous Serial Number: None

Principal Investigators: Louis H. Miller and James A. Dvorak

Other Investigators: Tsugiye Shiroishi

Cooperating Units: Dr. John Durocher, Walter Reed Army Institute of Research

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 16/12 | | | 16/12 |
| Professional | 4/12 | | | 4/12 |
| Other | 12/12 | | | 12/12 |

Project Description:

Objectives: To understand the mechanism by which the malarial merozoite gains entry into the red cell.

Methods Employed: Schizont infected red cells of Plasmodium knowlesi were separated from plasma, platelets and uninfected red cells. Uninfected red cells were then added to the schizonts, and the mixture was placed in a Dvorak chamber at 35°C. Culture media was perfused over the cells and rupture and reinvasion of red cells was observed by a Zeiss inverted phase microscope. Quantification of invasion was performed in Giemsa stained thin smears of the cells from the chambers. Uninfected red cells placed in the chamber included red cells of different species and enzymatically altered red cells. The influence of red cell membrane glycoprotein on invasion was studied.

Major Findings: 1) Observation of the interaction between merozoites and red cells. The interaction in the chamber appeared to be completely by

chance as the free merozoites moved within the chamber by energy supplied by the rupture process, the perfusion of fluid and Brownian movement. Merozoites did not appear to have motility, at least prior to contact with red cells. When the merozoite came in contact with a rhesus red cell, there was a marked folding and distortion of the red cell as the merozoite moved within the folds of the red cell. The red cell either engulfed the merozoite or the merozoite pushed itself within the red cell. Multiple invasion of red cells sometimes caused lysis of the red cells. The red cell ghost was spherical with merozoites attached to the outer surface of the plasma membrane rather than within the red cell. Merozoites attached to schizont infected red cells without invasion or any visible change in the red cell.

2) Host preference of merozoites in vitro. Red cells from man and night monkeys (Aotus trivirgatus), which are susceptible to infection by P. knowlesi, were invaded by P. knowlesi merozoites, although to a lesser degree than rhesus monkeys. Red cells from rats, mice, hamsters, rabbits, dogs and chickens, which are normally resistant to infection by P. knowlesi, were only rarely invaded. The merozoites in these cultures were healthy as they could attach to other infected monkey red cells.

3) The effect of enzyme alteration of red cell surfaces on P. knowlesi invasion. Red cells were treated with neuraminidase, pronase and trypsin before the cells were placed in the culture system. The enzyme effect was assayed by cell electrophoresis which measured the change in surface charge. Invasion of monkey red cells was uninfluenced by treatment of cells with neuraminidase, trypsin or pronase. Neuraminidase treated rat cells had no increased susceptibility to P. knowlesi merozoites. Whereas trypsin and neuraminidase had no influence on invasion of human red cells, pronase completely blocked invasion. Since pronase removed both sugars and peptides from the surface of red cells, the exact receptor on the red cell which interacts with merozoites is not defined. It is also unclear why pronase blocks invasion of human red cells and has no influence on invasion of monkey red cells.

4) Effect of human red cell glycoprotein on merozoite invasion. Human red cell glycoprotein isolated by lithium diiodosalicylate did not prevent invasion of human or monkey red cells at concentrations of 100 to 500 μg/ml. Negative experiments do not exclude glycoprotein receptors, since the glycoprotein isolated by this method is aggregated rather than monodispersed.

Significance to Bio-medical Research and the Program of the Institute:

Merozoites are extracellular for a short time before entering the red cell. During this period the parasite is vulnerable to antibodies which could agglutinate merozoites, lyse merozoites or block the receptor sites for the red cell membrane. An understanding of the mode of invasion and the chemical groups involved in the process could help in developing a vaccine

similar to the vaccines for influenza. Chemical changes in the parasitic membranes or receptor might explain antigenic variation.

Proposed Course of the Project: 1) To determine receptor site on red cell which the parasite recognizes and to elucidate the factors which culminate in invasion. 2) To study host specificity for P. falciparum. 3) To study antigenic variation in the in vitro system. 4) To study the ultrastructure of the merozoite-red cell interaction.

Publications:

Miller, L. H.: "Malaria" pp. 1113-1125. Edited by P. D. Hoeprich, "Infectious Diseases." Harper and Row, 1972.

Miller, L. H., Chien, S., and Usami, S.: Decreased deformability of Plasmodium coatneyi - infected red cells and its possible relation to cerebral malaria. Amer. J. Trop. Med. Hyg. 21: 133-137, 1972.

Miller, L. H.: The ultrastructure of red cells infected by Plasmodium falciparum in man. Trans. Roy. Soc. Trop. Med. Hyg. 66: 459-462, 1972.

Miller, L. H., Cooper, G. W., Chien, S., and Fremount, H. N.: Surface charge on Plasmodium knowlesi and P. coatneyi-infected red cells of Macaca mulatta. Exp. Parasit. 32: 86-95, 1972.

Miller, L. H.: Comments on lower-animal models. In Basic Research in Malaria, ed. E. H. Sadun. Helm. Soc. Washington, D.C. 39: 321-322, 1973.

Serial No. NIAID 132-B
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Immunity and Pathology of Malaria and Other Parasitic Diseases

Previous Serial Number: Same

Principal Investigators: Kendall G. Powers and Louis H. Miller

Other Investigators: John F. Finerty, P. C. C. Garnham (Fogarty International Scholar) and M. Abdel-Wahab

Cooperating Units: Laboratory of Microbial Immunity, NIAID, Imperial College Field Station, Ascot, England and Howard University

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 20/12 | 1/12 | 1/12 | 22/12 |
| Professional | 11/12 | 1/12 | 1/12 | 13/12 |
| Other | 9/12 | | | 9/12 |

Project Description:

Objectives: To study the immune response and pathological consequences of acute and chronic malaria and other parasitic diseases.

Methods Employed:

1. Various host-parasite models are used in these studies including Plasmodium knowlesi and P. cynomolgi in rhesus monkeys, P. falciparum in owl monkeys and P. berghei and P. vinckei in rodents.

We are attempting to identify and characterize different malarial antigens and their respective antibodies in order to determine their relative role in the overall immune response. Most of our current efforts are

concentrated on antigens associated with the membrane of malaria-infected erythrocytes. Of particular interest is the separation of these malarial antigens from the surface of infected erythrocytes so that they may be purified and used in various immunologic techniques to identify their function in malarial immunity. Enzymes such as trypsin, pronase and neuraminidase are being used for this purpose. We also are studying the chemistry of these membranes using electron dense colloids. Antigenic variation in malaria is being investigated using the schizont agglutination test to monitor antigenic differences between parasite populations during acute and chronic infections in a single nonhuman primate host. Rosettes, formed by parasites adhering to lymphocytes, are being studied to determine the mechanism involved in this reaction. Particular emphasis is placed on determining the stage of infection when rosettes are formed and the specificity of the reaction.

2. The immunologic response of mice to schistosome eggs injected in the presence of a concomitant P. berghei infection was investigated. Groups of mice received either P. berghei alone, P. berghei and Schistosoma mansoni eggs injected intravenously through the tail vein, or intravenous S. mansoni eggs alone. Mice were killed at various intervals after injection and the cellular immune response of the host to the schistosome eggs was determined by measuring the size of granulomata formed around the eggs in the lungs. Sera was also collected when each batch of mice was killed, and antibody specific for schistosome eggs antigen was measured using the soluble antigen fluorescent antibody test (SAFA).

3. It has been suggested that malarial parasites are much more infective to mosquitoes in the night than in the day. This hypothesis was examined using P. cynomolgi in rhesus monkeys and several strains of anopheline mosquitoes. Respective groups of mosquitoes were allowed to feed at midday and midnight during the early days of the acute infection. These mosquitoes were dissected 7 or 8 days later and the number of oocysts (an index of gametocyte infectivity) in each batch of 33 or more mosquitoes was recorded.

Major Findings:

1. A schizont agglutination test is being investigated as a method for studying the host immune response in malaria. Two antigenic preparations are used in this procedure, 1) erythrocytes infected with P. knowlesi schizonts and 2) infected erythrocytes that have been lysed by ammonia chloride. Agglutination occurs when these antigens are mixed with serum from chronically infected monkeys. Unlysed preparations were found to be highly specific and capable of differentiating a number of antigenic variants within a particular strain of malaria. A succession of antigenically distinct populations occurred during a chronic infection which might account for the parasite being able to persist and recrudesce in an otherwise immune host. When sera from such a chronically infected animal was titered against each of four homologous antigenic variants we recorded maximum titers of 1:500,000. Much lower titers (1:90 and less) were recorded when these same sera were

tested against the same four heterologous antigens. Ultrastructure studies of agglutinated material indicated that the infected erythrocytes were agglutinated at various protruding areas (spurs) of the cell membrane. Lysis of the infected erythrocytes resulted in a change in the antigenic response of the parasite preparation. Whereas the unlysed material was very specific for antibodies stimulated by a particular parasite strain or variant, lysed material cross-reacted with antibodies produced against vivax, ovale and falciparum infections in man and cynomolgi and knowlesi infections in monkeys. There also was an indication that lysed material was able to detect antibodies earlier than unlysed preparations when homologous sera were tested. These data suggest that antigens on the surface of infected cell might be different from those which are present on the schizont. Trypsin is being used in an attempt to separate some of these antigens from the surface of the infected erythrocyte membrane. Red cells treated with trypsin are no longer active in the schizont agglutination test. If we are indeed able to separate specific antigens from infected cell membranes then we should be able to collect, purify and identify the antigen responsible for antigenic variation.

Rosette formation in malarial infections is being investigated as another method to study the host immune response. BALB/c mice and rhesus monkeys infected with P. berghei and P. knowlesi, respectively, are being used in this study. Leukocytes are classified as rosette forming cells when 3 or more parasites adhere to them. The optimal condition for this reaction to occur was found to be 4°C for 30 minutes. Rosettes were only formed with extracellular trophozoites or schizonts and about 20% of the leukocytes in mice with 6, 7, or 8 day P. berghei infections were able to form these rosettes (see NIAID 117-C for details).

2. We investigated the immunosuppressive action of P. berghei on S. mansoni granuloma formation in mice as indicated by the size and cellular composition of the granulomata in the lungs. Our findings suggest a suppressive effect by the malaria on the host's immunologic response to schistosome eggs. This effect started as early as the 4th day after egg injection and reached its maximum by the 16th day, which more or less coincided with the peak of malaria parasitemia. These data suggest that the presence of malaria parasites played a principal role in suppressing the cellular reaction around the schistosome eggs. Concomitant malarial infections did not, on the other hand, influence humoral immunity in mice injected with schistosome eggs. There was no statistically significant difference in the level of antibodies specific for these eggs in the sera of mice with concomitant malaria infections than in mice injected with the schistosome eggs alone.

3. We were able to corroborate Hawking's theory that malaria parasites were more infective to mosquitoes in the night than in the day. Five controlled experiments were performed to test the validity of the theory of nocturnal infectivity of gametocytes of Plasmodium. Two gave equivocal results probably because synchronicity of parasitemia was absent; the remaining three, in which a langur strain of P. cynomolgi was used, largely confirmed Hawking's work and in particular demonstrated the much greater production of oocysts after night as compared with day feedings.

Significance to Bio-medical Research and the Program of the Institute:

During the malarial infection, the host produces antibodies to infected red cells that are coated with malarial antigens. Probably of equal importance for functional immunity are the antigens present on the surface of merozoites. Circulating antibody, with or without complement, could effectively destroy the merozoite or block its infectivity to erythrocytes.

If there are antigenic variants in malaria, as Brown and others suggest, then the problem of immunization becomes more difficult, even if the immunogens can be identified. It is hoped that these studies will develop methods to classify the antigenic variants, to separate and identify the antigens on the surface of the infected erythrocyte and to identify the stage of the parasite responsible for malarial immunity.

Proposed Course of the Project: Work on the schizont agglutination test will be continued; however, we plan to concentrate much of our investigations on P. falciparum in Aotus trivirgatus. Our principal studies will involve the identification and characterization of the antigenic components that are apparently on the erythrocyte surface.

Honors and Awards: K. G. Powers

Vice-President, Helminthological Society of Washington.

Vice-President, Tropical Medicine Association of Washington, D.C.

Member, American Society of Parasitologists Committee on Translations.

Chairman, NIAID Safety Committee.

Presented and invited seminar on malaria to the Survival School, NAS, Pensacola, Fla.

Publications:

Powers, K. G. and Cheever, A. W.: Comparison of geographic strains of Schistosoma mansoni in the rhesus monkey. Bull. Wld. Hlth. Org. 46: 301-309, 1972.

Cheever, A. W. and Powers, K. G.: Schistosoma mansoni infection in rhesus monkeys: comparison of the course of heavy and light infections. Bull. Wld. Hlth Org. 46: 295-300, 1972.

McMaster, P. R. B., Powers, K. G., Finerty, J. F. and Schiffman, G.: Tolerance to type III pneumococcal polysaccharide in monkeys. Immunol. Comm. (In Press).

Garnham, P. C. C. and Powers, K. G.: Periodicity of infectivity of plasmodial gametocytes: The Hawking Phenomenon. Inter. J. Parasit. (In Press).

Miller, L. H. and Dvorak, J. A.: Visualization of red cell membranes of lysed malaria infected cells by differential interference microscopy. J. Parasit. (In Press), 1973.

Serial No. NIAID 132-C
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Experimental Chemotherapy of Malaria and Other Protozoan Diseases.

Previous Serial Number: Same

Principal Investigator: Kendall G. Powers

Other Investigators: Masamichi Aikawa, Philip R. B. McMaster and Milford N. Lunde

Cooperating Units: Case Western Reserve University and Laboratory of Microbial Immunity, NIAID.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 8/12 | | | 8/12 |
| Professional | 5/12 | | | 5/12 |
| Other | 3/12 | | | 3/12 |

Project Description:

Objectives: To establish and maintain a series of models for use in evaluating potential antimalarial activity of various compounds. This includes parasites from birds, rodents and non-human primates. Drug activity is evaluated against both blood and tissue stages of the parasite. Compounds are also tested against other protozoan diseases when promising drugs become available.

Methods Employed: Compounds with antimalarial activity are first tested orally and intraperitoneally in mice infected with Plasmodium berghei and in certain instances in mice infected with P. vinckei. Efficacy is based on the percentage of infected erythrocytes in treated mice as compared to untreated controls. Compounds may also be evaluated against strains of

P. berghei resistant to chloroquine, pyrimethamine, quinine, DDS or certain long-acting sulfonamides and against P. vinckei resistant to pyrimethamine and chloroquine. When activity warrants, compounds are evaluated against blood-and sporozoite-induced P. gallinaceum in chickens and against P. cynomolgi, P. knowlesi and/or P. coatneyi in rhesus monkeys.

An additional host-parasite system is currently being utilized to further evaluate compounds that have demonstrated antimalarial activity. Aotus trivirgatus infected with chloroquine-resistant and normal strains of P. falciparum are being used to study the mode of action and chemotherapeutic activity of active compounds in the search for drugs that are efficacious against chloroquine-resistant falciparum malaria in man.

In certain instances promising compounds are evaluated against Toxoplasma gondii in mice. Various drug regimens are administered at different times during the acute infection and the results are compared with drugs currently being used in the chemotherapy of human toxoplasmosis. Compounds found to be active against this stage are tested further against cysts formed during the chronic stage of infection.

Major Findings: The effect of clindamycin (U-21) and N-demethyl-4'-pentyl clindamycin (U-24) was investigated in mice infected with the RH strain of Toxoplasma gondii. The drugs were administered by gavage or mixed in the diet for 1 to 14 consecutive days after injection of trophozoites. All mice were cured when given by gavage 50 mg/kg body weight of U-24 for 8 days or 400 mg/kg of U-21 for 11 days. While these drugs were uniformly curative when therapy was begun on the day of infection, the cure rate diminished when the treatment period was shortened or when the first dose was delayed for several days. Similar results were obtained when U-21, at a concentration of 200 mg percent, and U-24 at a concentration of 50 mg percent, were given ad libitum in the diet for 14 days. These analogues were also tested against the chronic stage of infection. Some antitoxoplasmic activity was detected for both compounds against the cysts of the RH strain but they both were inactive against cysts of the C37 strain of T. gondii (see Report NIAID 125-P and 125-A).

Clindamycin has also been shown to be active against chloroquine-resistant falciparum malaria in owl monkeys (see previous report NIAID 132-C). We are currently involved in a series of experiments in rhesus monkeys to study ultrastructural changes that occur in P. knowlesi as a result of treatment with clindamycin. It is hoped that these studies will give us an insight on the mode of action of this compound. Our preliminary results have demonstrated that the drug primarily affects the ribosomes and these changes are most prominent in the late trophozoites and schizonts. The effect is characterized by electron translucent areas in the center of the ribosome. With time the effect progresses and the ribosome appears to disintegrate resulting in irregular, amorphous areas in the cytoplasm. There also appear to be some secondary changes in the nuclei and mitochondria as a result of drug action. Affected nuclei seemed condensed and fibrilluar in appearance

while mitochondria were enlarged with prominent DNA fibers.

Significance to Bio-medical Research and the Program of the Institute:

There is a world-wide interest in the discovery of effective compounds for the treatment of chloroquine-resistant falciparum malaria in man. Particular emphasis has been placed on identifying drugs that differ chemically and in mode of action from commonly used antimalarials. The chlorinated lincomycin analogues are most promising compounds in this regard. Clindamycin is of particular interest since it is relatively nontoxic and is already commercially available for use in humans as an antibacterial agent. The curative activity of this drug against acute toxoplasmosis in mice suggests that this antibiotic may be of value in the therapy of acute toxoplasmosis in humans, possibly even against toxoplasma infections acquired during pregnancy in which the use of sulfadiazine and pyrimethamine is questioned because of their toxicity.

Proposed Course of the Project: Studies on the use of clindamycin in combination with other compounds such as sulfadiazine and pyrimethamine are being considered to determine if such combinations will be more effective than clindamycin alone against acute and chronic toxoplasmosis in mice. As a result of our findings on the mode of action of clindamycin against P. knowlesi in rhesus monkeys we are contemplating additional experiments using P. falciparum in owl monkeys

Publications:

McMaster, P. R. B., Powers, K. G., Finerty, J. F. and Lunde, M. N.: The effect of two chlorinated lincomycin analogues against acute toxoplasmosis in mice. Am. J. Trop. Med. Hyg. 22: 14-17, 1973.

Serial No. NIAID 132-D
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Drug Resistance in Experimental Malaria

Previous Serial Number: Same

Principal Investigator: Richard L. Jacobs

Other Investigators: None

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 6/12 | | | 6/12 |
| Professional | 3/12 | | | 3/12 |
| Other | 3/12 | | | 3/12 |

Project Description:

Objectives: To investigate the mechanisms of acquired drug resistance in rodent malarial parasites; to develop and characterize different drug-resistant strains by their cross-resistant patterns to various antimalarial drugs, pathogenicity, morphological changes, and stability of resistance in the absence of selective drug pressure.

Methods Employed: Different strains of Plasmodium berghei are maintained in mice by blood transfer. Gametocyte-producing strains are maintained by cyclical passage through A/J mice, hamsters and Anopheles stephensi. Prior to the selection of resistant strains, standard dose-suppression tests were conducted to establish the sensitivity of the parent strain of P. berghei to the various drugs under study. Parasitemia was followed in blood smears stained by the Giemsa procedure. The suppressive effect of different doses of drug was determined by comparison of the mean percent parasitemia in treated groups with the untreated control group of mice.

The procedure used to develop drug-resistant strains of P. berghei involved repeated blood passages of the parasite in groups of mice treated with the highest dose of drug that allowed for some development of parasitemia during each passage. Transfer to fresh mice was normally made by day 7, using large numbers of parasitized erythrocytes (up to 20 million per mouse) which were inoculated intraveneously. Drugs dosages were increased as indicated by parasite development in the previous passage in order to maintain the maximum degree of selective drug pressure. Parasite strains were considered totally resistant when they developed in mice treated with the maximum tolerated dose (MTD) of the drug. Resistant strains were subsequently maintained in mice treated with the MTD of drug during each passage. Drugs were given orally (stomach tube) to mice on days 0,1,2, and 3 after inoculation of parasites. In order to assess the stability of resistance in the absence of selective drug pressure, the resistant strains were subpassaged repeatedly in untreated mice and tested periodically for drug sensitivity, changes in morphology, virulence and other characteristics associated with resistance.

Major Findings: A strain of P. berghei has been developed that is totally resistant to clindamycin, that is, it develops in mice treated with 1000 mg/kg/day which is the MTD of clindamycin for mice. This totally resistant strain represents a 40-fold increase in resistance since the parent strain was almost suppressed by doses of 25 mg/kg/day. The clindamycin-resistant strain is normally sensitive to chloroquine, quinine, pyrimethamine, and minocycline. No obvious differences in morphology or virulence have been observed in this resistant strain as compared to the parent strain. The stability of resistance to clindamycin in the absence of drug pressure is currently being assessed. After 10 successive untreated passages over a period of 71 days, no decrease in resistance has been observed.

Total resistance to minocycline has not been developed at the present time. After 49 passages in mice treated with minocycline, over a period of 336 days, the strain of P. berghei tolerates daily doses of 500 mg/kg. This represents about a 5-fold increase in resistance since the parent strain develops in mice treated with 100 mg doses. The partially resistant strain appears to be normally sensitive to clindamycin. No changes have been observed in parasite morphology or virulence as resistance to minocycline increased. This strain is being subpassaged in minocycline treated mice in an attempt to increase the degree of resistance.

These results on development of resistance to antibiotics by P. berghei indicate that, in the case of clindamycin and minocycline, resistance develops less readily than resistance to the commonly used antimalarials such as chloroquine, quinine, pyrimethamine, or primaquine. Also, resistance to one antibiotic does not confer cross-resistance to the other or cross-resistance to other antimalarial drugs tested.

Significance to Biomedical Research and the Program of the Institute:

Drug-resistant malaria, particularly resistance to chloroquine, continues to present a formidable problem in the chemotherapy of malaria. Strains of P. falciparum that are resistant to all of the commonly used synthetic antimalarials, as well as to quinine, continue to be encountered in widely separated areas of the world. Also, other species of malaria that infect man may become refractory to presently effective drugs. The appearance of drug resistance in malarial parasites of man has served to emphasize the need for a better understanding of the basic mechanisms of drug action on the parasite and other aspects of host-drug-parasite relationships. Plasmodium berghei has proven to be a valuable laboratory model for the investigation of the mode of action of various antimalarial compounds and some of the basic aspects of acquired drug resistance.

Recent reports regarding the successful use of antibiotics, especially clindamycin and tetracycline, for the treatment of drug resistant malaria in man raise questions regarding the prolonged usefulness of these compounds. If no cross-resistance occurs, then resistance to one will not invalidate the usefulness of the other compound. Studies in mice infected with P. berghei should give some insight regarding the ease of development of resistance to these compounds, cross-resistance patterns and stability of resistance once it has been developed.

Proposed Course of the Project: This project will be continued to develop complete resistance to minocycline. Complete cross-resistance patterns will be established between the two resistant strains, both to the antibiotics and to the conventional antimalarials. Stability of resistance in the absence of drug pressure will be investigated.

Serial No. NIAID 132-E
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Labelling of Sporozoites of Malaria

Previous Serial Number: Same

Principal Investigators: Richard L. Jacobs and Louis H. Miller

Other Investigators: Robert W. Gwadz and Louis C. Koontz

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 20/12 | | | 20/12 |
| Professional | 11/12 | | | 11/12 |
| Other | 9/12 | | | 9/12 |

Project Description:

Objectives: To develop methods for labelling sporozoites in order to determine their distribution in the mammalian host (kinetics) and the development from the sporozoite through the exoerythrocytic stages within the parenchymal cells of the liver. The effect of drugs and immunity on the kinetics of sporozoite distribution and the developing EE forms will be investigated. Labelled sporozoites will provide a useful tool for the study of relapse mechanisms.

Methods Employed: The NK-65 strain of Plasmodium berghei is maintained in cyclical passage through mosquitoes (Anopheles stephensi), mice and hamsters. P. cynomolgi is maintained by cyclical passage in rhesus monkeys and different species of mosquitoes (A. maculatus and A. freeborni). Sporozoites are harvested by dissection of infected mosquito salivary glands at appropriate times after the infective blood meal. Developing sporozoites are labelled in vivo by feeding tritiated compounds to infected mosquitoes. The degree of radioactive isotope incorporated into sporozoites is determined by autoradiography. Viability of labelled

sporozoites is assessed by intraveneous injection into susceptible hosts. The development of the radioactive sporozoite in the mammalian host will be followed in tissue sections and in dissociated liver cells by autoradiography. The distribution of the labelled sporozoites will be studied by scintillation counting of tissue homogenates of various organs.

Major Findings: Procedures have been worked out for the radioactive labelling of developing sporozoites of P. berghei and P. cynomolgi using either of several tritiated purines (adenine, adenosine, deoxyadenosine, deoxyguanosine). The tritiated pyrimidines that have been tested to date (thymine, thymidine, orotic acid) were not incorporated into sporozoites as determined by autoradiography using exposure periods as long as 90 days. Tritiated adenine fed to infected mosquitoes in syrup solution at levels of 5, 10 or 25 μCi/ml resulted in well labelled sporozoites with little or no apparent effect in their infectivity. The feeding of higher levels of tritiated adenine suppressed both sporozoite production and infectivity of sporozoites. The tritiated purines are incorporated in both nuclear and cytoplasmic regions of sporozoites. The degree of labelling, as evidenced by the numbers of grains associated with individual sporozoites on autoradiographs, was related both to the level of isotope fed to mosquitoes and to the time of exposure to the photographic emulsion. When low levels of isotope were fed, both the percentage of sporozoites labelled and the average degree of labelling can be increased by extending the time of exposure to the photographic emulsion - which ordinarily ranges from about 7 to 21 days.

Significance to Biomedical Research and the Program of the Institute:

The greatest gap in our knowledge of the life cycle of the malaria parasite involves the mechanism by which relapses occur. The generally accepted explanation involving the reinvasion of hepatic cells by merozoites liberated from the exoerythrocytic cycle is not proven. More recently, it has been suggested that relapses may result from some sporozoites that remain dormant for long periods of time within the hepatic parenchemal cells before developing into exoerythrocytic forms. To date, these latent forms have not been demonstrated, possibly because of their small size and similarity to the cytoplasm of liver cells in stained preparations. It is hoped that the present study will determine whether late EE forms in P. vivax and P. ovale are due to slow development from sporozoites or from invasion of merozoites from the primary EE form to the secondary EE forms. The results of this study should be of great importance in the chemotherapy of hepatic schizonts (causal prophylaxis and radical cure).

Proposed Course of the Project: These studies will be continued with major emphasis on the location of labelled sporozoites in tissue and following the development of labelled sporozoites in the mammalian host, especially in monkeys infected with P. cynomolgi, which is similar to P. vivax in man.

Serial No. NIAID 132-0
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Pathogenesis of disease (Chagas' disease) resulting from infection with Trypanosoma cruzi

Previous Serial Number: Same

Principal Investigator: Franklin A. Neva

Other Investigators: Dr. Gustavo Parajon of Hospital Baustista in Managua, Nicaragua

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 10/12 | | | 10/12 |
| Professional | 1/12 | | | 1/12 |
| Other | 9/12 | | | 9/12 |

Project Description:

Objectives: To elucidate some of the factors that determine why infection with T. cruzi is associated in some individuals with either acute or chronic disease. To develop improved serologic procedures for diagnosis of infection and/or disease due to T. cruzi.

Methods Employed: A number of strains of trypanosomes are maintained in culture, others by syringe passage, and some are maintained in serial passage alternating between mice and vector bugs. The biologic characteristics of strains kept in mouse-bug passage are also tested at intervals by checking peripheral parasitemia and histopathology of mice. Colonies of two species of triatomid bugs are maintained. One strain of T. cruzi is passaged in cell cultures of embryonic beef tissue, and tissue phases of the parasite (mainly amastigotes and trypomastigotes) are harvested for antigen. Complement-fixation and gel-diffusion tests for detection of antigen-antibody reactions are done in a standard manner. Antisera to

different T. cruzi antigen preparations are obtained by immunization of animals and by collection from patients. Follow-up examinations of patients surveyed earlier in the field have been conducted by Dr. Parajon for clinical evaluation and diagnosis of their eardiac status.

Major Findings:

Field Study in Nicaragua: The status of the final clinical evaluation of the Nicaraguan cases who were being followed-up is not clear because communications with Dr. Parajon in Managua were cut off after the December 1972 earthquake. It has been learned that Dr. Parajon survived the earthquake but the status of records pertaining to the study is not known; the hospital where Dr. Parajon worked and where the records may have been kept was destroyed in the earthquake. Attempts are being made to re-establish contact so the clinical evaluation phase of the study can be completed. Results of the serologic studies are being prepared for publication.

Antigen from T. cruzi from infected cell cultures: The cross-reactions of our cell culture T. cruzi antigen by CF test with sera from certain patients with muco-cutaneous leishmaniasis appears to be due, at least in large measure, to contamination of the antigen with beef cells from the cultures. Three of the six antigen preparations which showed this cross-reactivity contained the equivalent of an estimated 400,000 or more beef cells per ml at the working dilution of antigen. Antigens with less than this number of beef cells did not cross-react. In addition, sera from some cases of American leishmaniasis were reactive by CF with beef cell antigens alone. With Ouchterlony gel-diffusion tests multiple precipitin lines were demonstrated when sera from T. cruzi patients were reacted vs different T. cruzi antigens. With at least some of the sera it appears that some distinctly different antigens are present in material from infected tissue cultures as compared to conventional epimastigote antigens while other antigens are shared. Gel-diffusion techniques appear to be promising for analysis of T. cruzi tissue culture antigens.

Comparative behavior of different strains of T. cruzi: These studies still elude manuscript form and submission for publication, but analysis of histopathology of different strains in mice has started. Meanwhile, different strains of T. cruzi that have maintained have been made available to other investigators in LPD (Luban and Mercado) for their studies.

Significance to Biomedical Research and the Program of the Institute:

The tissue culture antigen for serodiagnosis of T. cruzi infection appears to be equal to or greater in sensitivity to the best available antigen prepared from crithidia and hitherto considered a standard antigen. More study of the specificity of the tissue culture antigen is still needed. There is reason to believe that the tissue culture antigen might have some advantages to the conventional antigens in selected instances. Our field

experience in Nicaragua has provided a model for evaluation of the importance of T. cruzi infection as a cause of heart disease in communities. Even though our study in Nicaragua had deficiencies as far as precision is concerned, it has served to provide a basis that may be recommended in the future for other groups and countries to assess the significance of this parasite as a public health problem.

Proposed Course of the Project: If necessary, a trip will be made to Nicaragua to stimulate Dr. Parajon to complete the clinical evaluation of the field study. There still is hope that the studies on different strains of T. cruzi will be put together for publication. The use of gel-diffusion techniques for comparative analysis of T. cruzi antigens appears promising and will be pursued - especially to determine whether antigens from amastigotes and trypomastigotes are unique. This finding could have important implications for improved diagnosis of T. cruzi infection and possibly in differentiating patients who are infected from those with actual disease.

Publications:

Neva, F.A. American Trypanosomiasis. Diseases Transmitted From Animals to Man. 6th Ed. Edited by, Hubbert, W.T., McCulloch, W.F., and Schnurrenberger, P.R. Publisher, Charles C. Thomas (In Press)

Serial No. NIAID 133
1. Parasitic Diseases
2. Malaria - Primate and Human Malaria
3. Chamblee & Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Susceptibility of Anopheline Mosquitoes to Simian and Human Malaria Infections and Their Ability to Transmit to Simian and Human Hosts

Previous Serial Number: Same

Principal Investigators: William E. Collins and Louis H. Miller

Other Investigators: Richard H. Glew and David J. Wyler

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 77/12 | | | 77/12 |
| Professional | 5/12 | | | 5/12 |
| Other | 72/12 | | | 72/12 |

Project Description:

Objectives: (1) To determine the relative susceptibility of anopheline mosquitoes to simian and human malaria infection; (2) to determine the rate and degree of parasite development within various mosquito hosts; (3) to attempt transmission of the various simian malarias to a wide spectrum of simian hosts by the bites of infected mosquitoes; and (4) to provide mosquitoes infected with human malaria for transmission studies in prison inmate volunteers.

Methods Employed: Isolates of simian malaria are maintained either in monkey hosts or in the frozen state for use in transmission studies. Malaria species under study include Plasmodium brasilianum (2 strains), P. cynomolgi (9strains), P. coatneyi, P. fieldi (3 strains), P. simiovale, P. simium, P. fragile (2 strains), P. gonderi, P. knowlesi (4 strains), P. inui (17 strains), P. jefferyi and P. hylobati. Studies are also made

on P. falciparum (7 strains), P. vivax (13 strains) and P. malariae maintained in monkeys. Various species of Anopheles (A. maculatus, A. stephensi, A. freeborni, A. atroparvus, A. albimanus, A. quadrimaculatus and A. balabacensis balabacensis) mosquitoes are allowed to feed on infected simian and human hosts. Infection potential to a given mosquito species of a given malaria strain in a variety of hosts is determined and transmission by direct mosquito bites are made. Occasionally, when numbers of mosquitoes are low and gland dissections are positive, attempts to infect monkeys are made by intravenous inoculation of sporozoites from gland dissections.

Major Findings: The results of the transmission studies with the various malarias are as follows:

P. knowlesi - H strain: Transmission was obtained to M. mulatta on 13 occasions with A. b. balabacensis mosquitoes. The prepatent periods ranged from 5 to 6 days with a mean of 5.4 days. Transmission to Aotus trivirgatus was obtained twice. Prepatent periods wer 16 and 27 days.

P. cynomolgi bastianellii: Transmission was obtained on 40 occasions with A. maculatus, A. freeborni and A. b. balabacensis mosquitoes. The prepatent periods ranged from 7 to 13 days with a mean of 8.4 days.

P. gonderi: Transmission was obtained on 9 occasions with A. maculatus, A. freeborni and A. b. balabacensis mosquitoes. Prepatent periods ranged from 11 to 15 days with a mean of 12.7 days.

P. inui - Mulligan strain: Transmission was obtained once with A. b. balabacensis mosquitoes. The prepatent period was 11 days.

P. falciparum - Panama II strain: Transmission from one monkey to another was obtained on 7 occasions with A. freeborni mosquitoes. The prepatent periods ranged from 19 to 67 days with a mean of 31.4 days.

P. falciparum - Burma Thau strain: Transmission from one monkey to another was obtained twice with A. freeborni mosquitoes. The prepatent periods were 19 and 24 days.

P. vivax - Salvador II strain: Transmission was obtained twice with A. albimanus and twice with A. freeborni mosquitoes. The prepatent periods were 19, 55, 23 and 34 days respectively.

The successful transmission of two different strains of P. falciparum from one Aotus monkey to another via the bites of infected mosquitoes indicated the usefulness of this system for studies on the complete cycle of this very important human malaria in a simian model. Studies on the susceptibility of different sub-species of Aotus trivirgatus indicated that A. t. trivirgatus was refractory to some strains of P. falciparum and only partially susceptible to others as compared to the A. t. griesiamembra

monkeys. In addition, even though infections with P. vivax were similar in each sub-species, mosquito infection was more readily obtained by feeding on infections in the latter.

A comparison of the susceptibility of different strains of A. albimanus from Central America with regard to infection with P. vivax indicated that differences existed between two strains from El Salvador and one from Panama. Strains appear to be more susceptible to infection with parasites from adjacent areas. The successful transmission of the Salvador II strain by A. albimanus from El Salvador indicated the compatibility of the native vector and native P. vivax parasites from that country.

Studies on the course of parasitemia in Aotus monkeys indicated that recurring recrudescences of infections of P. vivax were infectious to mosquitoes for over one year. With P. falciparum, however, infections of any consequence were only obtained during the first peak in the parasitemia. In several sporozoite induced infections with P. falciparum, the initial parasitemias were of a low magnitude. However, each recrudescence resulted in higher parasitemias and eventual death of the animals in from 3 to 4 months.

Significance to Bio-medical Research and the Program of the Institute:

This project is part of an integrated program of the Laboratory of Parasitic Diseases in the study of simian malaria in monkeys and its relationship to man and human malaria. A clear-cut delineation of the parasite-vector relationship is essential for varied species of simian malaria, simulating all of the human species in life pattern and morphology, to carry on significant studies on the chemotherapy, immunology and other aspects of malaria research. A full exposition of the host-parasite-vector relationships of the available monkey parasites is a necessity in providing these useful host-parasite combinations. The availability of infections of human malarias in monkeys makes it possible to study the effect of drugs on these parasites and on the ability of these parasites to infect different mosquitoes. It serves also as a source of P. vivax, P. malariae, and P. falciparum infected mosquitoes for use in other related investigations.

Proposed Course of the Project: These studies will be conducted and expanded to include the evaluation of a number of anopheline species of mosquitoes in relation to their potentials as vectors of species of simian and human malaria. Efforts will continue to be directed towards obtaining large numbers of gland positive mosquitoes of several simian species of malaria for use in inoculating monkeys for tissue parasite studies and to challenge immunized monkeys. Efforts will be continued towards developing more effective and efficient means not only of producing larger numbers of mosquitoes for laboratory transmission studies but also of increasing the longevity of such mosquitoes.

Publications:

Collins, W E., Contacos, P.G., Skinner, J.C., and Guinn, E.G.: Studies on the transmission of simian malaria. V. Infection and transmission of the B strain of Plasmodium cynomolgi. J. Parasit., 58: 653-659, 1972.

Collins, W.E., and Contacos, P.G.: Transmission of Plasmodium falciparum from monkey to monkey by the bite of infected Anopheles freeborni mosquitoes. Trans. Roy. Soc. Trop. Med. & Hyg., 66: 371-372, 1972.

Collins, W.E., Contacos, P.G., Krotoski, W.A., and Howard, W.A.: Transmission of four Central American strains of Plasmodium vivax from monkey to man. J. Parasit., 58: 332-335, 1972.

Collins, W.E., Contacos, P.G., Guinn, E.G., and Skinner, J.C.: Plasmodium simium in the Aotus trivirgatus monkey. J. Parasit., 59: 49-51, 1973.

Collins, W.E., Contacos, P.G., Guinn, E.G., and Richardson, B.B.: The influence of splenectomy on infections of Plasmodium fieldi in Macaca mulatta monkeys. J. Parasit., 59: 41-48, 1973.

Collins, W.E., Contacos, P.G., Stanfill, P.S., and Richardson, B.B.: Studies on human malaria in Aotus monkeys. I. Sporozoite transmission of Plasmodium vivax from El Salvador. J. Parasit. (In Press).

Collins, W.E., Neva, F.A., Chaves-Carballo, E., Stanfill, P.S., and Richardson, B.B.: Studies on human malaria in Aotus monkeys. II. Establishment of a strain of Plasmodium falciparum from Panama. J. Parasit. (In Press).

Serial No. NIAID 133-A
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies of the Exoerythrocytic Stages of Primate Malaria

Previous Serial Number: NIAID 133-A and NIAID 133-F

Principal Investigators: William E. Collins

Other Investigators: None

Cooperating Units: Dr. Thomas M. Sodeman, University of Michigan

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 12/12 | | | 12/12 |
| Professional | 1/12 | | | 1/12 |
| Other | 11/12 | | | 11/12 |

Project Description:

Objectives: (1) To describe the exoerythrocytic (EE) stages of species and strains of malaria in which they have not previously been described; (2) to study the histochemical reaction of EE bodies; (3) to study the prophylactic and curative effects of antimalarial drugs on the EE stages of malaria parasites and to determine the morphologic alterations within the EE stages due to antimalarial drugs.

Methods Employed: Exoerythrocytic forms are produced in a new host by transmission through a mosquito vector from a previously infected host. This is accomplished: (1) by mosquito bite; (2) by direct inoculation of the sporozoites into the liver; or (3) by intravenous inoculation of sporozoites.

Livers are biopsied on selected days after exposure or following drug therapy. Biopsies are processed after paraffin imbedding, as frozen sections or by freeze substitution. In addition to Giemsa staining, fluorescein labeled anti-sera are used to identify exoerythrocytic forms in tissue sections obtained at biopsy at appropriate intervals following direct intrahepatic inoculation of viable sporozoites. Adjacent sections are stained with suitable histochemical agents for morphologic identification and description.

Major Findings: Thirty-nine liver biopsies were made in two species of non-human primates in attempts to find exoerythrocytic (EE) stages of five species of malaria parasites. Five of these were shown to contain EE bodies.

Exoerythrocytic schizonts of three simian malarias were identified by indirect immunofluorescence in liver biopsy material fixed in Carnoy's solution and imbedded in paraffin up to nine years previously. Three, four, and five day EE schizonts of P. cynomolgi, B strain, were identified using immunofluorescent techniques.

A concerted effort (18 biopsies) was made to find and describe the EE stages of P. gonderi. Only the seven day forms were found.

Significance to Bio-medical Research and the Program of the Institute:

Since the earliest use of antimalarial chemotherapeutic agents, it has been recognized that relapses often occur in previously treated patients. It is now understood that these relapses occur because of the release of forms from the exoerythrocytic stages, these stages having been resistant to the therapy. Therefore, it is extremely important that the activity of any new candidate antimalarial drug against the exoerythrocytic stages be known. Previously, the number of host-parasite models available for the study of this drug activity in primates has been limited. The current project is providing the necessary information and techniques to enable investigators to utilize a number of additional parasite species and primate hosts to evaluate the activity of candidate drugs against pre-erythrocytic stages of the parasite.

Proposed Course of the Project: (1) The demonstration of yet undescribed exoerythrocytic stages of the different primate malarias; (2) the study of the morphologic effects of new antimalarial drugs on the EE bodies. In collaboration with Dr. T. M. Sodeman of the University of Michigan, biopsy material is being studied by electron microscopy to determine the ultrastructure of the EE bodies and other stages of different species of primate malaria.

Publications:

Schnitzer, B., Sodeman, T. M., Mead, M. L., and Contacos, P. G.: Pitting function of the spleen in malaria: Ultrastructural observations. Science, 177: 175-177, 1972.

Sodeman, T. M., Contacos, P. G., Collins, W. E., Smith, C. S., and Jumper, J. R.: Antimalarial studies on the prophylactic and radical curative activity of RC-12. Bull. Wld. Hlth. Org., 47: 425-428, 1972.

Krotoski, W. A., Jumper, J. R., and Collins, W. E.: Immunofluorescent staining of exoerythrocytic schizonts of simial plasmodia in fixed tissue. Am. J. Trop. Med. Hyg., 22: 159-162, 1973.

Schnitzer, B., Sodeman, T. M., Mead, M. L., and Contacos, P. G.: An ultrastructural study of the red pulp of the spleen in malaria. Blood, 41: 207-218, 1973.

Collins, W. E., Contacos, P. G., Jumper, J. R., Smith, C. S., and Skinner, J. C.: Studies on the exoerythrocytic stages of simian malaria. VIII. Plasmodium knowlesi. J. Parasit., (In Press).

Krotoski, W. A., Collins, W. E., and Jumpber, J. R.: Detection of early exoerythrocytic schizonts of Plasmodium cynomolgi by immunofluorescence. Am. J. Trop. Med. Hyg., (In Press).

Serial No. NIAID 133-B
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immuno-ecology of Primate Malaria

Previous Serial Number: Same

Principal Investigators: William E. Collins

Other Investigators: None

Cooperating Units: Central America Malaria Research Station, El Salvador

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 13/12 | | | 13/12 |
| Professional | 3/12 | | | 3/12 |
| Other | 10/12 | | | 10/12 |

Project Description:

Objectives: To explore the total ecology of malaria and to correlate circulating antibodies with the presence of parasites and to orient both of these factors with transmission cycles, level of endemicity, and clinical disease.

Methods Employed: Blood samples, on filter paper, are collected from human populations in areas endemic for malaria. These specimens are mailed back to the central laboratory in Chamblee, Georgia for determination of fluorescent antibody titers. The accompanying field studies vary in scope, but wherever possible the following investigations are carried out: (1) Giemsa-stained blood films of the human population for parasite rates and species identification; (2) surveys for malaria parasites in mammals other than man; and (3) studies on the anopheline fauna, including identification, seasonal distribution, biology and malaria infection rate.

Major Findings: The fluorescent antibody studies in a low incidence area of El Salvador were completed. Serologic profiles accurately reflected the past malarial experience in people surveyed. In a study of six villages in Costa Rica, it was found that active transmission was either absent or at a very low level. Malaria was more likely to be associated with old or imported cases rather than with current local transmission. Positive titers to P. malariae indicated that although current transmission is either absent or at a low level, the parasite was still present in the population.

Approximately 230 members of a population under insecticide protection were cured of their infections with chloroquine and primaquine. After six, 12 and 18 months, the same individuals were examined both serologically and parasitologically. Since a rise in IFA titer is indicative of recent experience with malaria, it was possible to determine the percentage of the population which experienced infection or reinfection during each interval. The new statistic, the positive serologic conversion index, may prove to be a very useful epidemiological tool in malaria control programs.

A very serious outbreak of P. falciparum in Escobal, Republic of Panama, was investigated both during the height of the epidemic and 8 months later. School age children were examined both serologically and parasitologically. It was found that although there was a good correlation between infection and antibody response during the outbreak, after a period of only eight months, all the antibody titers had dropped to negative levels. Thus, the IFA technique could not be of value in determining the extent of a previous epidemic in a population normally considered free of infection.

Significance to Bio-medical Research and the Program of the Institute:

Immunological studies using the FA technique offer an opportunity to determine prior experience with malaria of populations in areas endemic for malaria. The opportunity to correlate field studies with laboratory investigations greatly expands our potential for study on the basic problems associated with human malaria.

This type of investigation gives insight into the mechanisms of the development of protective responses in populations naturally infected with malaria. Information derived from these studies will eventually enable us to predict the future course of malaria in areas of high endemicity and possibly to alert health authorities on the developing potential of epidemics.

Proposed Course of the Project: Cooperative studies will be continued with the Central America Malaria Research Station, El Salvador. These investigations will be expanded to other areas of Africa and South America as our techniques improve and the occasions arise.

Publications:

Collins, W. E., and Skinner, J. C.: The indirect fluorescent antibody test for malaria. Am. J. Trop. Med. Hyg., 21: 690-695, 1972.

Warren, McW., Collins, W. E., and Jeffery, G. M.: Sero-epidemiology of malaria. Revista del Instituto de Investigaciones Medicas de El Salvador. 2: 58-69, 1973.

Serial No. NIAID 133-C
1. Parasitic Diseases
2. Primate & Human Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Fluorescent Antibody Studies in Simian and Human Malaria

Previous Serial Number: Same

Principal Investigator: William E. Collins

Other Investigators: None

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 3/12 | | | 3/12 |
| Professional | 1/12 | | | 1/12 |
| Other | 2/12 | | | 2/12 |

Project Description:

Objectives: (1) To determine the development and long-term persistence of specific antibody in malaria-infected humans and monkeys; (2) to study the immunologic relationships between the different species of human and simian malarias; and (3) to determine the effect of biological and surgical immunosuppression on the development and persistance of antibody reactions.

Methods Employed: Serum samples are taken from patients and animals prior to infection and at regular intervals thereafter to determine by fluorescent antibody methods the rate of specific antibody production and the persistence and titer of antibody during the months and years following termination of the malaria infection. Animals having past malarial infection are challenged with heterologous species and strains of Plasmodium sp. and the immunologic response studied by the fluorescent antibody technique. Animals are given curative and subcurative treatments with antimalarial drugs and the effect measured using the FA technique. Animals splenectomized or otherwise surgically treated before and after infection are studied

in regard to the FA response. Sera from patients infected with malaria are studied using the specifically labeled immune globulins to determine the patterns of response to each.

Major Findings: Most of the work with IFA and monkeys was done in conjunction with project 133-D which involved the determination of antibody levels subsequent to immunization and challenge with malarial antigens and parasites.

The persistence of malarial antibody was determined in 55 volunteers infected with Plasmodium vivax via sporozoite inoculation. The average length of the primary parasitemia was only 7.9 days. Antibody titers rose to a peak mean IFA titer of 1:202 between 11 and 20 days of patent parasitemia and thereafter dropped quite rapidly. Although all individuals reached an IFA titer of 1:40 or greater, by 60 days, some individuals had negative titers and by 6 to 8 months, the mean IFA titer was only 1:13. Upon relapse of the infection, however, titers rose to very high levels and were maintained there much longer. It appears that a short primary infection may result in a low transient IFA response, but that repeated infection may result in high sustained antibody titers.

Significance to Bio-medical Research and the Program of the Institute:

There continues to be a need for additional information on the immune response of the primate host to malaria. The immunological studies using the fluorescent antibody technique offer an opportunity to measure the effect of prior experience with malaria on current clinical attack and to determine the presence of malarial experience in an endemic population. The FA studies may also provide additional criteria for grouping the different strains, subspecies and species of primate malaria.

Proposed Course of the Project: Studies will continue on the development and persistence of the antibody response in monkeys and man infected with different species of malaria and to determine the effect of multiple infection and chemotherapy on antibody response. Further efforts will be made to determine the effect of various immunization procedures on the antibody response in conjunction with project 133-D.

Publications:

Collins, W. E., Skinner, J. C., Contacos, P. G., Guinn, E. G., and Richardson, B.: The influence of splenectomy on infections of Plasmodium fieldi in Macaca mulatta monkeys. J. Parasit., 59: 41-48, 1973.

Serial No. NIAID 133-D
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunological Studies on Primate Malaria

Previous Serial Number: Same

Principal Investigators: William E. Collins

Other Investigators: None

Cooperating Units: Dr. Peter G. Contacos, Center for Disease Control, Atlanta, Georgia

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 21/12 | | | 21/12 |
| Professional | 1/12 | | | 1/12 |
| Other | 20/12 | | | 20/12 |

Project Description:

Objectives: (1) To evaluate the possibilities for effective biologic prophylaxis against malaria; (2) to analyze the antigenic composition of various species and/or strains of simian malaria; and (3) to localize (insofar as possible) the morphological and/or antigenic unit(s) principally responsible for specific immunofluorescent reactions following naturally acquired malarial infections.

Methods Employed: For the antigenic analysis and the active immunization studies, purified parasite suspensions are obtained from monkeys at the time of peak parasitemia. Packed parasitized erythrocytes are treated with saponin. The parasites are then washed repeatedly with buffered saline. The packed parasites are either stored at -70°C or in the lyophilized state. The parasites are crushed in a Hughes tissue press and the antigenic components extracted by mixing with dilute salt solutions. Another method utilized in the preparation of antigen is to repeatedly freeze and thaw the parasitized

erythrocytes. The parasitic and erythrocytic stroma are removed by centrifugation, and the soluble malarial antigens used for various purposes. The antigens prepared are studied by gel filtration chromatography. In addition, parasitized erythrocytes treated with formalin (0.1%) are used as antigen in immunization experiments. Monkeys are actively immunized with protein extracts, with intact parasitized erythrocytes, or with intact parasites freed from the red blood cell by lysis, with fractions of hemolysates from parasitized erythrocytes, or with residue of extracted ruptured parasites. After a suitable period of time, the monkeys are challenged with malaria. Presence of antibody in the serum of the immunized animals is confirmed by indirect FA and immunoelectrophoresis. Antigenic composition of the various preparations is studied by immunoelectrophoresis. Passive immunization is studied by administering immune globulin to monkeys in a dose of 100 mg/kg. The gamma globulin is prepared from blood obtained from monkeys evidencing a degree of immunity to malaria. The ion-exchange batch technique is utilized in preparing relatively pure gamma globulin. The gamma globulin is given prior to exposing the monkeys to bites of heavily infected mosquitoes and at 15 day intervals for 3 doses. Fractionation of hemolysates from parasitized erythrocytes and aqueous extracts of parasites is accomplished by column gel filtration.

Major Findings: A total of twenty-four Macaca mulatta monkeys were immunized with a heat stable (100°C) serum soluble antigen (SSA-S) of P. knowlesi given in 1 to 6 doses at concentrations of from 10 to 120 milligrams. Six of 10 control monkeys died as a result of the infection. Monkeys immunized with 50 milligrams or greater of the vaccine had a death rate similar to that of the control. However, in those animals given 10 to 40 milligrams of the vaccine, the death rate was only 30 percent. It appears that much smaller dosages may be effective.

Since monkeys have been protected by immunization with formalinized parasitized erythrocytes, a study was made using P. cynomolgi and M. mulatta monkeys immunized with various extracts of formalinized parasitized erythrocytes after being crushed in a Hughes press. Although the results were inconsistent, some of the animals immunized with the peak II of the aqueous extract (after passage over a G-100 Sephadex column) and the non-soluble residue had greatly reduced average daily parasitemias.

There have been no significant changes from our previous observations that immunization of monkeys using a variety of immunizing materials does not completely protect from infection.

Significance to Bio-medical Research and the Program of the Institute:

Until recently the availability of reliable and effective antimalarial chemotherapy obviated the necessity for intensive immunologic studies relating primarily to biologic prophylaxis against malaria. However, with the advent of some strains of falciparum malaria resistant not only to all the accepted synthetic antimalarials but also to quinine, the need for such

studies became obvious. The effectiveness with which passive or active immunization will protect monkeys against infection, not only to the homologous malaria parasite but more especially to the heterologous parasite, should indicate the feasibility of effective biologic prophylaxis against malaria in man.

Proposed Course of the Project: The studies will be continued with further emphasis on separation of antigenic fractions by means of column chromatography. These individual fractions will then be tested for their immunizing ability against malaria. Emphasis will continue to be placed on serum soluble antigens as immunizing agents as well as immunization by irradiated sporozoites.

Publications:

Collins, W. E., and Contacos, P. G.: Immunization of monkeys against Plasmodium cynomolgi by X-irradiated sporozoites. Nature New Biology, 236: 176-177, 1972.

Serial No. NIAID 133-E
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Study of Cell-mediated Immunity (CMI) in Simian Malaria

Previous Serial Number: None

Principal Investigator: David J. Wyler

Other Investigators: William E. Collins

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 8/12 | | | 8/12 |
| Professional | 8/12 | | | 8/12 |
| Other | | | | |

Project Description:

Objectives: To investigate the development and specificity of both in vivo (skin test) and in vitro (lymphocyte transformation) correlates of cell-mediated immunity in simian malaria.

Methods Employed: Peripheral lymphocytes from control and infected (with P. cynomolgi B) rhesus monkeys are cultured in vitro without antigen and in the presence of an aqueous extract of crushed P. cynomolgi B parasites or PHA. The incorporation of tritiated thymidine after five days of culture is determined, as an index of the relative degree of lymphocyte blastogenesis stimulated by antigen or PHA. The course of this parameter in sporozoite- and blood-induced malaria infections will be followed over several periods of patency and inter-patency, to determine the time-course of development and occurrence (if any) of cellular immunosuppression during patent parasitemia. This in vitro study will be complemented by a parallel in vivo study (in the same subjects) of skin testing using the same antigen preparation. Species specificity and relative potency of several antigenic preparations will be investigated.

Major Findings: The appropriate culture conditions for in vitro cultivation of rhesus peripheral lymphocytes have been partially determined, and the appropriate antigen concentration providing optimal lymphocyte transformation has been established. In a few preliminary studies, we have been able to demonstrate lymphocyte blastogenesis in infected monkeys using malaria antigen.

Significance to Bio-medical Research and the Program of the Institute:

A thorough understanding of immune response to malarial infection requires investigation of not only humoral mechanisms ("B" cell function) but also cellular immune mechanisms ("T" cell function) which may have a role independent of B cell function or may augment antibody response (helper role). Unfortunately, little work in this area has been done in primate malaria. Knowledge in this area may also have important implications for vaccination against malaria as well as understanding immunosuppression which occurs in this disease.

Proposed Course of the Project: Comparison of several antigen preparations from P. cynomolgi B (ether extracted fraction, serum soluble antigen, unprocessed parasite) will be made to determine which preparation is most potent as a specific mitogen in our system. Antigen preparations from other simian malaria (e.g.: P. coatneyi) will be tested with lymphocytes from monkeys infected with P. cynomolgi B to determine species specificity of skin test and lymphocyte transformation response. Several monkeys infected with P. cynomolgi B (both sporozoite and blood-induced infections) will be studied over several months to determine the course of these parameters of CMI during infection.

Serial No. NIAID 133-F
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The role of malaria in the induction of lymphoma by Epstein-Barr virus (EBV).

Previous Serial Number: None

Principal Investigators: Franklin A. Neva, Louis H. Miller, and David J. Wyler

Other Investigators: William E. Collins

Cooperating Units: M. A. Epstein, Department of Pathology, University of Bristol, Bristol, England

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 2/12 | | | 2/12 |
| Professional | 1/12 | | | 1/12 |
| Other | 1/12 | | | 1/12 |

Project Description:

Objectives: (1) To determine whether lymphoma can be induced in monkeys by inoculation with EBV and malaria parasites; (2) to determine what role malaria plays in the oncogenesis of Burkitt's lymphoma; (3) to establish an *in vitro* culture line of any tumor that so arises.

Methods Employed: Three groups of owl monkeys (*Aotus trivirgatus*) were inoculated with EBV-containing lymphoblastoid cells, either on the same day as, or nine or twenty-five days following blood-induced infection with *Plasmodium simium*. Baseline and multiple post-inoculation serum specimens were obtained for determination of titers of anti-EBV antibodies. Daily parasite counts were made.

A control study, to determine if EBV is oncogenic in the owl monkey in the absence of concomitant malaria infection is being studied elsewhere by one of the investigators (Epstein).

Major Findings: Of a total of twelve owl monkeys studied, seven have died, of which four were autopsied. Gross and microscopic examination of these monkeys failed to reveal any tumors.

Significance to Bio-medical Research and the Program of the Institute:

Epidemiologic evidence has supported the notion that malaria plays a role in the etiology of African Burkitt's lymphoma, in conjunction with EBV. To determine the validity of this theory, an experimental model is needed. If such a model can be found, and if the relation of malaria and EBV to Burkitt's lymphoma is demonstrated in this model, important understanding of viral oncogenesis can result.

Proposed Course of the Project: The five remaining monkeys will be followed indefinitely for evidence of development of lymphoma. Monkeys that die will be autopsied, and any suspicious tissue that is suggestive of tumor will be cultured *in vitro* and studied by light and electron microscopy.

Serial No. NIAID 133-G
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Gametogenesis and the Infectivity of Primate Malarias to Mosquitoes

Previous Serial Number: None

Principal Investigators: Robert W. Gwadz

Other Investigators: William E. Collins

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Othe NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 3/12 | | | 3/12 |
| Professional | 2/12 | | | 2/12 |
| Other | 1/12 | | | 1/12 |

Project Description:

Objectives: To determine what factors regulate gametogenesis and the viability of gametes in various human and simian malarias.

Methods Employed: The viability and infectivity of gametes of Plasmodium sp. are being studied in various anopheline mosquitoes and in vitro with particular reference to "gamete-specific" host immunity. Circadian rhythms of gametocyte infectivity and longevity are being investigated with round-the-clock mosquito feedings.

Major Findings: Plasmodium falciparum infections in Aotus monkeys produce non-infectious gametocytes during recrudescences in spite of continually increasing parasitemias and gametocytemias. In these infections, the early stages of the sporogonic cycle (exflagellation, fertilization and ookinete formation) do not occur in vitro, or in vivo in the gut of a normally susceptible mosquito host.

Significance to Bio-medical Research and the Program of the Institute:

The full utilization of human malaria / Aotus monkey models for research has been hampered by the frequent non-infectivity of gametocytes to mosquitoes. This project proposes to determine the basis for this lack of infectivity and improve existing model systems necessary for the study of sporogony and sporozoite-induced infections.

Proposed Course of the Project: This recently initiated project will continue and be expanded to include the development of methods to improve the infectivity of gametocytes for the laboratory infections of mosquitoes.

Serial No. NIAID 133-H
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effects of Chemosterilization on the Development and Transmission of Malaria Parasites in Mosquitoes

Previous Serial Number: None

Principal Investigators: Mohamed S. Omar and William E. Collins

Other Investigators: Robert W. Gwadz

Cooperating Units: Entomology Research Division, Agriculture Research Service, USDA, Gainesville, Florida

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 7/12 | | | 7/12 |
| Professional | 2/12 | | | 2/12 |
| Other | 5/12 | | | 5/12 |

Project Description:

Objectives: To study the effects of chemosterilants on the development of malaria parasites in various anopheline vectors and to determine the rate of malaria transmission by chemosterilized mosquitoes.

Methods Employed: Various malaria parasites are used in this study including the simian parasites, Plasmodium cynomolgi, P. gonderi in rhesus monkeys; the human malaria parasite, P. vivax in owl monkeys. Sterilized mosquitoes used for transmission studies are Anopheles freeborni, A. quadrimaculatus, A. maculatus and A. albimanus. Mosquitoes are sexually sterilized by dipping pupae for 60 minutes in 1% aqueous solution of the chemosterilant p,p,-bis (1-azirdinyl)-N-methyl phosphinothioic amide (ENY-61585). Pupae are then removed from the sterilizing solution and rinsed with water before being placed in emergency containers.

Major Findings: The preliminary results indicate that there is a reduction in the rate of infection in sterilized mosquitoes when compared to the untreated controls fed simultaneously on infected monkeys. This was also accompanied by a reduction in oocyst counts per infected gut in the treated mosquitoes compared with the relevant controls. Studies are now being conducted to characterize the nature of this effect on the various stages of the sporogonic cycle in the mosquito and to test the viability of sporozoites, when present, in the salivary glands of treated mosquitoes.

Significance to Bio-medical Research and the Program of the Institute:

One of the promising aspects in biological control of mosquitoes is the genetic manipulation of insect populations. One of these approaches is the sterile-male release technique involving sterilization and release of laboratory-bred males to compete with wild males in the field for mating with female mosquitoes. Sterilization of mosquitoes could be achieved either by X-radiation or by the use of chemosterilants. If the chemosterilant used for treating males could also be used at the appropriate dose level to sterilize female mosquitoes and likewise disrupt or prevent the development of malaria parasites within the mosquito host, its impact would have obvious advantage in genetic control operations. Thus, instead of the exclusive release of sterilized males, sexually sterilized females could also be released in the field without the potential hazards of increasing the incidence of malaria transmission.

Proposed Course of the Project: This project will be completed within the current year.

Serial No. NIAID 133-I
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Comparative Histological and Histochemical Studies on the Sporogonic Cycle of Malaria Parasites

Previous Serial Number: Same

Principal Investigators: Mohamed S. Omar and William E. Collins

Other Investigators: None

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 2/12 | | | 2/12 |
| Professional | 2/12 | | | 2/12 |
| Other | | | | |

Project Description:

Objectives: To study (1) morphological or histological differences between the development of the parasite in mosquitoes showing different degrees of susceptibility to plasmodial infections; (2) histochemical demonstration of the relationship between the mosquito tissue and the malaria parasite.

Methods Employed: Histological: Plasmodium sp. were studied in laboratory bred mosquitoes (Anopheles balabacensis balabacensis, A. maculatus and A. freeborni) fed on infected rhesus monkeys (Macaca mulatta). Various stages of the sporogonic cycle were demonstrated on smears from the stomach or in paraffin sections made of infected mosquitoes at appropriate time intervals after the infective blood meal. Giemsa or Haematoxylin-eosin stains were applied for staining. Histochemical: Conventional histochemical methods for the detection of nucleic acids (DNA & RNA), polysaccharides, and proteins were systematically used on paraffin-embedded sections. Tests for

enzymes are carried out on fresh or fixed cryostat sections. Immunofluorescent staining using the IFA technique were also employed on infected mosquito sections previously fixed in Carnoy's solution for detection of oocysts. Recently, autoradiographic techniques are being applied to determine the uptake of various tritium-labelled nucleic acid precursors by malaria parasites in infected mosquitoes which are fed on sugar solutions of the radioactive compounds.

Major Findings: Sections of mosquito guts infected with malaria parasites showed distinct differential reaction intensity in various organs and tissue of the mosquito and the parasite. These variations were not only observed at various stages of development of the parasite within the mosquito, but also in the different Anopheles species with various degrees of susceptibility to the same Plasmodium. Histochemical observations indicated also that there are three distinct regions of secretory activity in the salivary glands of female mosquitoes as judged by differential staining reactions. Enzyme tests carried out on cryostat sections showed that strong acid and alkaline phosphatase activites were present in the apices of the midgut epithelial cells. However, the degree of the corresponding activities of these enzymes in oocysts on the mosquito gut differs from strong positive in the early stage of development to variable or negative in mature oocysts. Malaria oocysts of P. cynomolgi were readily identified by indirect immunofluorescent technique (IFA) in Carnoy's fixed and paraffin sectioned infected mosquitoes. Groups of mosquitoes which were previously fed on various concentrations of tritium-labelled compounds in 5% Karo syrup (5, 25 and 50/ μC/ml) for various time intervals were fixed and embedded in paraffin and are ready for autoradiographic examination.

Significance to Bio-medical Research and the Program of the Institute:

The incidence of malaria in its many ecological environments is primarily dependent on those factors which bring a suitable mosquito vector in contact with the susceptible host. Although it has been recognized for many years that not all anophelines are capable of transmitting malaria, the reasons for this are, in many instances, unclear. These studies will not only assist in an understanding of the reasons for mosquito susceptibility to malaria, but more importantly, in what makes a species a vector.

Proposed Course of the Project: This project will be completed in the current year.

Publications:

Krotoski, W. A., Omar, M. S., and Jumper, J. R.: 1973. Immunofluorescent staining of plasmodial oocysts in the mosquito. J. Parasit. (In Press).

Serial No. NIAID 133-J
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Gametocytocidal and Sporontocidal Effects of Some Commonly Used Antimalarial Drugs on the Sporogonic Cycle of Malaria Parasites

Previous Serial Number: Same

Principal Investigators: Mohamed S. Omar and William E. Collins

Other Investigators: None

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 13/12 | | | 13/12 |
| Professional | 8/12 | | | 8/12 |
| Other | 5/12 | | | 5/12 |

Project Description:

Objectives: To investigate the effect of commonly used and experimental antimalarial drugs on gametocytes and sporogony of malaria parasites; to characterize the mode of action of the drugs on the early stages of plasmodia in the mosquito vector (exflagellation, fertilization, ookinete formation, penetration of the gut and encystment).

Methods Employed: Blood-induced infections of the parasite, Plasmodium cynomolgi (B strain) in rhesus monkeys was used in these studies. Drug solutions or suspensions were administered to infected monkeys via the oral route. Batches of Anopheles maculatus were allowed to fed on infected monkeys immediately before treatment, at various hours on the first day of treatment, and at daily intervals thereafter. The effect of the drug on the developing stages in the mosquito was examined in stomach smears and mosquito sections made at appropriate time intervals after the infective blood meal.

In some cases, the effect of the drug was also investigated by feeding the compound directly to infected mosquitoes along with the sugar solution.

Major Findings: Investigations to determine the gametocytocidal and sporontocidal effects of the commonly used antimalarial agents, primaquine, chlorguanide, pyrimethamine, chloroquine, mepacrine and quinine have been completed. During the last year, studies were mainly directed to characterize the activities of some experimental drugs which have been shown to have potential antimalarial action. These are the pyrocatechol compound, RC-12, the phenanthrenemethanol WR 33,063, the quinolinemethanol WR 30,090 and of the 1-aminocyclopentanecarboxylic Acid WR 14,997.

A. RC-12: Single or repeated doses of RC-12 at 25 mg. base/kg./day were found to have substantial sporontocidal activity against P. cynomolgi in mosquitoes fed 4 to 8 hours after initial drug administration. Retarded or degenerate oocysts were detected in mosquitoes fed 24 till 72 hours after treatment began. However, no sporozoites were detected in the salivary glands of these mosquitoes. From the available observations of drug activity it appears that RC-12 acted primarily by sterilizing the gametocytes in the blood and rendering them non-infective to mosquitoes. However, the gametocytocidal action of the drug was less apparent compared to its rapid sporontocidal effect. The tested doses caused the disappearance of parasites from the blood within 3-4 days of treatment. The results obtained indicated also that a single dose treatment of RC-12 (25 mg. base/kg.) was quite sufficient to completely interrupt mosquito infection 4 hours after drug administration and subsequently. These results confirm the views that doses of RC-12 are retained in the body at sufficient levels for several days when given in one oral dose. In contrast, when RC-12 (0.1% base in 10% sucrose solution) was fed directly to infected mosquitoes ad lib, no apparent sporontocidal effect was observed. These data suggest that there is a difference in the mode of action of RC-12 depending on whether the drug is given to the vertebrate or invertebrate host. Interestingly enough, the salivary glands of mosquitoes which had ingested the drug directly with the sugar solution showed gross histopathological changes, became swollen and reaching 3-4 times the size of the normal glands.

B. Phenanthrenemethanol WR 33,063 and Quinolinemethanol WR 30,090: Dosages with WR 33,063 (31.6 mg/kg/day) and WR 30,090 (10 mg/kg/day) were administered to infected monkeys once daily for 7 consecutive days. Although the tested regimens had eliminated parasitemia from the blood within 3-4 days after the beginning of therapy, mosquito infectivity was observed as long as gametocytes were present in the peripheral blood. It appears that neither compound has any effect on gametocytes which were present or formed in the blood or on the sporogonic stages of the parasite in the insect vector. Therefore, it might be concluded that both drugs had no antimalarial activity against gametocytes and sporogony of P. cynomolgi.

C. 1-aminocyclopentanecarboxylic Acid WR 14,997: WR 14,997 was tested against P. cynomolgi at dose levels (60 mg/kg and 100 mg/kg/day) administered for 7 consecutive days. The results indicated that both drug regimens had no

effect, whatsoever, on the course of parasitemia in the monkey or on mosquito infectivity. During the period of the experiment, mosquitoes became moderately to highly infected when fed on either medicated or non-medicated control monkeys. On the other hand, when the drug was offered to infected mosquitoes along with the sugar diet (0.1% in 10% sucrose solution), it showed an appreciable sporontocidal activity against the developing oocysts. The latter forms showed various degrees of retarded development and degeneration. Mosquitoes proved negative for sporozoites in the salivary glands. In addition, the prophylactic activity of WR 14,997 was tested against P. cynomolgi in two monkeys. The animals received daily doses (60 mg/kg or 100 mg/kg) starting one day prior to and on the same day of infected mosquito bites and continued for 7 consecutive days thereafter. The treated animals along with an untreated control developed patent infections. These results suggest that WR 14,997 in the tested doses has no apparent prophylactic action against sporozoite-induced infections of P. cynomolgi in rhesus monkeys.

Significance to Bio-medical Research and the Program of the Institute:

The increasing occurrence of drug resistant strains of human malaria to the available antimalarial drugs has revived the interest in the search for new effective antimalarials. The evaluation of such candidate compounds with regard to their causal prophylactic, curative and sporontocidal capabilites would be of potential value in selecting those drugs with these types of activities for possible future use in human malaria. Also, in characterizing compounds capable of interrupting or preventing the sporogonic cycle of plasmodia in the mosquito vector would be of great value in malaria control programs.

Proposed Course of the Project: Project completed.

Publications:

Omar, M. S., Collins, W. E., and Contacos, P. G.: Gametocytocidal and Sporontocidal Effects of Antimalarial Drugs on Malaria Parasites I. Effect of single and multiple doses of primaquine against Plasmodium cynomolgi. Exptl. Parasit., (In Press), 1973.

Omar, M. S., and Collins, W. E.: The Antimalarial Activity of RC-12 Against Gametocytes and Sporogony of Plasmodium cynomolgi. Tran. Roy. Soc. Trop. Med. Hyg., (In Press), 1973.

Serial No. NIAID 133-K
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Genetics of Vector Capacity of Anopheline Mosquitoes for Primate Malarias

Previous Serial Number: None

Principal Investigators: Robert W. Gwadz

Other Investigators: William E. Collins and Mohamed S. Omar

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 9/12 | | | 9/12 |
| Professional | 8/12 | | | 8/12 |
| Other | 1/12 | | | 1/12 |

Project Description:

Objectives: (1) To select lines of mosquitoes susceptible or refractory to a particular species of primate malaria; (2) to determine the mode of inheritance of susceptibility to this particular malaria; (3) to determine susceptibility of the selected mosquito line or lines to various geographic strains of this malaria and to other species of human and simian malaria; (4) to determine what physiologic factors may be involved in rendering certain mosquitoes refractory or susceptible to infection with human and simian malarias.

Methods Employed: Colonies have been established from the mass-reared progeny of individual females of Anopheles albimanus susceptible to the Rio Meta (Columbia) strain of Plasmodium vivax. Sub-colonies are being established from individual susceptible females and are being maintained by mass-rearing or single pair inbreeding. The mode of inheritance of susceptibility is being determined by standard methods of genetic analysis.

Anopheles albimanus is highly refractory to both parasites in the laboratory but is known to transmit P. falciparum under field conditions. The mosquito gonadotropic cycle is being manipulated by blood-feeding mosquitoes before and/or after infection with malaria. In addition, the neuro-hormonal system of the mosquito host is being surgically and chemically altered to determine the roles this system may play in regulating parasite development.

Major Findings: Initial experiments have shown that susceptibility to a particular strain of P. vivax in A. albimanus responds to selection. The susceptibility of 4 lines of A. albimanus has been raised from approximately 30% to over 70% in one generation. Pre- and post-infection blood-feedings have been shown to have no effect on the insusceptibility of A. albimanus to infection with P. cynomolgi.

Significance to Bio-medical Research and the Program of the Institute:

Malaria is an entomological problem, but the genetic basis for vectorial capacity is poorly understood. Development of genetically defined lines of mosquito vectors will provide important tools for a variety of research efforts.

Susceptible and refractory lines will provide material for histochemical, physiological and bio-chemical investigations into the nature of the mosquito-malaria host-parasite relationship.

Refractory lines of mosquitoes should prove useful in biological control schemes involving vector displacement or genetic manipulation of vector populations.

Understanding of vector genetics will facilitate the analysis of populations of mosquito polymorphic with respect to vector capacity.

Proposed Course of the Project: These recently initiated studies will be continued and expanded to include a number of anopheline species and their relationships with a variety of human and simian malarias.

Serial No. NIAID 133-L
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Role of Serum Complement in Primate Malaria

Previous Serial Number: None

Principal Investigator: Richard H. Glew

Other Investigators: William E. Collins

Cooperating Units: John Atkinson and Michael M. Frank, Laboratory of Clinical Investigation

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 3/12 | | | 3/12 |
| Professional | 2/12 | | | 2/12 |
| Other | 1/12 | | | 1/12 |

Project Description:

Objectives: This study was designed to determine levels of serum complement (generally as C'4) during the course of infection in rhesus monkeys with Plasmodium coatneyi. In addition, an attempt was made to evaluate the possible protective role of serum complement in the host defenses against malaria by depleting the late components of serum complement through injections of cobra venom factor into the monkeys.

Methods Employed: After rhesus monkeys had been infected with P. coatneyi by blood inoculation, several series of serum samples were drawn during periods of schizont rupture (schizogony is equivalent to the clinical phenomenon of paroxysm in the human); levels of C'4 in these samples were then determined.

Infected monkeys received intraperitoneal injections of cobra venom factor in order to activate and deplete the late components of serum complement (C'3 through C'9). The level of parasitemia in the monkeys was

then checked frequently during the subsequent several days in order to determine whether or not complement depletion had any deleterious effect on host defenses in malaria.

Major Findings: Studies of C'4 levels in 8 infected monkeys demonstrated that beginning about 14 days after blood inoculation, each episode of schizont rupture was associated with a drop in C'4 of at least 30%. In general, schizogony resulted in a drop in C'4 of between 75% and 95%, when samples drawn just prior to onset of schizont rupture were compared with samples drawn serially during schizont rupture. The nadir in C'4 occurred about 4 to 8 hours after the onset of schizogony, generally around the time of peak parasitemia in that cycle. C'4 levels were back up to near-normal levels by about 30 hours after the onset of schizogony. C'4 levels changed little during schizogony until about two weeks after blood inoculation, or about the time when humoral immunity would be expected to have come into play. In addition, at any time late in infection when the peak parasitemia was less than about 25,000 parasites per $mm^3$ C'4 levels also did not change significantly.

Depletion of the late components of complement (as measured by C'6 levels) by means of a single intraperitoneal injection of cobra venom factor (CVF) into infected monkeys had no effect on parasitemia. Two monkeys received CVF in the pre-immune phase of logarithmically-rising parasitemia, and three monkeys received CVF during the steady state phase of chronic infection. CVF failed to alter the course of parasitemia in these animals.

Significance to Bio-medical Research and the Program of the Institute:

Little is known about the possible protective host response to malaria, other than that it is possible to passively transfer partial immunity to non-immunes with antibodies. This Unit has demonstrated transient decreases in serum complement in human volunteers experimentally infected with P. vivax, generally during relapses. This study confirms these findings in monkeys infected with P. coatneyi. More importantly, these monkeys were studied at much higher levels of parasitemia than the humans, and much more profound drops in complement were noted. All these results raise the possibility that serum complement might play a role in the immune response to malaria.

Our ability to follow infections in the monkeys for prolonged periods of time provides information that elucidates the fate of complement in malaria. In the first place, in animals chronically infected with P. coatneyi, C'4 levels did not decrease during schizogony if the parasitemia was at relatively low levels, suggesting that a certain critical amount of antigenic material must be released intravascularly during schizogony (in a partially immune host) to provide for complement consumption. Furthermore, since marked depletion of C'3 - C'9 by CVF had no effect on parasitemia, it is concluded that although complement consumption in malaria seems to be related to a partially immune state in the host,

the consumption is not a protective factor in the host response to malaria. Rather, it seems that complement is consumed as an immunological non-functional bystander in some unknown antigen-antibody reaction.

Proposed Course of the Project: Attempts will be made to elucidate the mechanism by which complement is consumed in malaria. One approach will be to attempt to demonstrate the appearance at the time of schizogony in infected monkeys of serum soluble antigens capable of combining with both antibody and complement.

Serial No. NIAID 133-M
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Induction of Quinine Resistance in P. falciparum in Aotus Monkeys

Previous Serial Number: None

Principal Investigator: Richard H. Glew

Other Investigators: Louis H. Miller and William E. Collins

Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 5/12 | | | 5/12 |
| Professional | 2/12 | | | 2/12 |
| Other | 3/12 | | | 3/12 |

Project Description:

Objectives: Experimental induction of quinine resistance in plasmodia has been accomplished only in P. berghei (in mice) and P. relictum (in canaries). However, studies in human volunteers by this Unit suggested that the Colombia (KSN) strain of P. falciparum acquired decreased sensitivity to quinine during sequential courses of increasing days of quinine therapy. Accordingly, this study was instituted in an attempt to induce increasing quinine resistance in P. falciparum in Aotus monkeys.

Methods Employed: Owl monkeys (Aotus trivirgatus griseimembra) were infected by the intravenous inoculation of infected blood with P. falciparum, Panama 11 strain. The original infection was the result of 6 serial blood passages, without exposure to antimalarial drugs, beginning with blood from an infected boy from the Republic of Panama.

Initially, infections were treated with progressively increasing courses of quinine (125 mg/kg daily by stomach tube in a single dose); serial courses progressed through 3, 5, 7, 10 and 14 days of treatment.

When an infection survived 10 or 14 days of therapy, and particularly when it continued smear positive through 14 days of treatment (R11 resistance) these infections were subpassaged to other animals by blood for additional treatment.

Major Findings: Of 4 animals infected with parasites not previously exposed to drug, 3 apparently were cured by treatment with quinine for 14 days (2 animals died 2 weeks after completion of therapy and were smear negative at that time; a third monkey continues to be smear negative 3 weeks after treatment), while one exhibited R1 resistance to the regimen. Of 7 animals infected with parasites from the second, third or fourth serial generation under drug pressure, three exhibited R11 resistance to 14 days of quinine, two exhibited R1 resistance, and two were cured by treatment with quinine for 14 days.

Significance to Bio-medical Research and the Program of the Institute:

With the increase in multi-drug resistance of P. falciparum, quinine has become important in the treatment of drug-resistant strains. Quinine often is used in combination with slower acting blood schizontocidal drugs, in order to hold down parasitemia while the ancillary drugs effect a radical cure.

The work presented here indicates that under the proper conditions increasing resistance to quinine may occur. Development of decreased sensitivity to quinine seems to require a strain of P. falciparum with inherent moderate resistance to quinine, as well as drug pressure.

Proposed Course of the Project: Of first importance will be continued serial passage of this strain under drug pressure in an attempt to further increase its resistance to quinine, perhaps to the point where parasitemia increases during treatment with quinine for 14 days (R111 resistance). When maximal resistance has been obtained, attempts will be made to evaluate the stability of the quinine resistance during drug free passages (by blood), as well as stability of the resistance during passage through sporogonic cycles in the mosquito. Furthermore, studies will be made to determine if the resistance of this strain to other antimalarials has been altered by the development of increased resistance to quinine.

Serial No. NIAID 134
1. Parasitic Diseases
2. Malaria - Human Malaria
3. Atlanta, Georgia and Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Pathogenesis of the malarial paroxysm

Previous Serial Number: Same

Principal Investigators: Franklin A. Neva and Richard H. Glew

Other Investigators: Louis H. Miller and Michael Frank

Cooperating Units: Department of Justice, Bureau of Prisons, Federal Penitentiary, Atlanta, Georgia; Section on Medical Parasitology of Laboratory of Clinical Investigation; Dr. David W. Alling of Laboratory of Clinical Investigation.

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 6/12 | | | 6/12 |
| Professional | 3/12 | | | 3/12 |
| Other | 3/12 | | | 3/12 |

Project Description:

Objectives: (1) To define, with greater precision than has been done previously, the time relationship of parasite development to onset and subsequent elements of the malarial paroxysm and (2) to determine causative factors in the genesis of fever and other manifestations of the malarial paroxysm.

Methods Employed: Study of the time relationship of parasite development to the onset and subsequent elements of the malarial paroxysm is limited to patients with naturally occurring or induced malaria whose infections exhibit a highly synchronized cycle of parasite development and/or a well synchronized cycle of clinical signs and symptoms. Thin blood

smears are prepared throughout a selected paroxysm at 15 to 30 minute intervals while periodic blood specimens are taken for hematologic and serologic determinations, and body temperature is monitored. Subsequently, several observers independently perform parasite differential counts on each slide by recording total numbers of specified parasite forms observed per 80,000 RBCs.

Serum complement levels, assayed with a standardized 50% hemolytic endpoint technique, are measured at selected intervals early after the onset of patent malaria as well as late in the illness prior to treatment. When possible, a paroxysm during the primary attack as well as during a relapse is studied. Special effort is made to bracket well-defined fever paroxysms with complement determinations and correlate the serum complement levels with the immune status of the host, as measured by the IFA and CF techniques, and the parasite density.

Attempts to demonstrate a transferable pyrogen and/or other biologically active substances involves withdrawal of 250 or 500 ml of blood at a specified time during a well-synchronized paroxysm. The blood, withdrawn and handled using standard blood banking procedures, is re-infused in the same patient as whole blood or component fractions after the patient has been treated.

Major Findings: 1. After consultation and preliminary evaluation of data with Dr. Alling, it was decided that most accurate parasite quantification was best achieved by counting parasitized cells per 80,000 RBCs. This procedure assumes no significant loss in RBCs over one paroxysm (the loss in parasitized cells is $< 1\%$) and avoids relating the figure to another variable, such as numbers of WBCs. The actual calculations for rates of disappearance and appearance of different parasite stages have not yet been performed. Reasonably complete sets of slides are available on 8 paroxysms, and there are incomplete data from an additional 5 paroxysms.

2. Attempts to demonstrate a circulating pyrogen in the early stage of a malarial paroxysm were carried out in 5 cases of P. vivax malaria. In every instance the re-infusion of the same blood back into the same individual 3 or 4 days later failed to produce either fever or symptoms during the immediate post-infusion period.

3. The study of alterations of serum complement during the malarial paroxysm has been completed and is being prepared for publication. In 14 cases serum samples were obtained from 21 paroxysms during the primary attack and a total of 17 paroxysms were studied in 11 patients during relapse. In 10 of the 17 relapse episodes a significant depression in whole serum complement was found whereas only one of the paroxysms during the primary attack exhibited a similar fall in complement levels. Depletion of C4 was found in a few additional patients without a change in whole serum complement indicating that C4 may be a more sensitive indicator and that the classical complement pathway is involved. Complement depletion was dependent upon presence of CF antibody and was positively correlated with the degree of parasitemia.

Significance to Bio-medical Research and the Program of the Institute:

The further refinement in information relating the time-course of the parasite cycle to fever and other clinical manifestations of malaria is basic to further studies on the pathogenesis of the malarial paroxysm. If schizont rupture and merozoite release, for example initiate an immunologic reaction which, in turn, leads to fever or other consequences then fairly precise information on the sequence of events is needed. From the studies carried out in volunteers a sufficient background of information has developed so that some similar studies can be carried out in patients with naturally-occurring disease. A collaborative study on complement changes in monkeys with malaria was carried out with the Laboratory of Clinical Investigation (see Project No. NIAID 133-L).

Proposed Course of Project: The data must be analyzed in greater detail by the principal investigators--time has been the main limiting factor. The complement study in human cases will be submitted for publication. Further efforts are planned to demonstrate circulating antigen(s) during the paroxysm in sera of cases already studied. The possibility of investigating some aspects of complement utilization in an in-vitro system is being considered. Carefully selected patients with naturally occurring malaria may be studied at the Clinical Center in the future.

Serial No. NIAID 134-A
1. Parasitic Diseases
2. Human Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Evaluation of Antimalarial Drugs and/or Drug Regimens

Previous Serial Number: Same

Principal Investigators: Louis H. Miller, Richard H. Glew and David J. Wyler

Other Investigators: William E. Collins

Cooperating Units: Department of Justice, Bureau of Prisons

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 35/12 | | | 35/12 |
| Professional | 5/12 | | | 5/12 |
| Other | 30/12 | | | 30/12 |

Project Description:

Objectives: To evaluate standard antimalarial agents as well as new drugs with demonstrated antimalarial activity, and to develop acceptable regimens for their use in the treatment of human malaria.

Methods Employed: Healthy inmate volunteers at the U. S. Penitentiary, Atlanta, Georgia, are fully informed of the risks and discomforts of malaria, and are not subjected to coercion or excessive inducements. Volunteers are under the 24-hour care and supervision of the resident investigators. For the evaluation of chemotherapeutic agents, volunteers are exposed to the bites of several mosquitoes infected with malaria. Treatment is begun after patent parasitemia is demonstrated. Volunteers are followed with daily thick blood smears for at least 3 to 6 months after treatment.

Major Findings: For the past few years our major interest has been the evaluation of the efficacy of 7-chloro-lincomycin, or clindamycin. Earlier studies by this Unit had demonstrated that in six volunteers with sporozoite-induced P. vivax infections, treatment with clindamycin, 450 mg

orally every 6 hours for 7 days, cleared asexual parasites from the blood but had no activity against exoerythrocytic stages.

Since asexual parasitemia did not begin to decrease for 3 days after initiating therapy with clindamycin against nonmalignant malarias, quinine was given with clindamycin to rapidly reduce parasitemia in P. falciparum malaria. Infections with Rl chloroquine-resistant P. falciparum (Cambodian 1 and Malayan IV strains) in five volunteers were cured by quinine, 650 mg every 8 hours for 3 days, followed by clindamycin, 450 mg every 6 hours for 7 days. Furthermore, 3 days of quinine followed by only 3 days of clindamycin, (450 mg orally every 6 hours) cured 7 volunteers infected with Rll and Rlll chloroquine-resistant P. falciparum (Panama ll and Burma Thau strains).

In order to diminish the total length of treatment required with this regimen, it seems desirable to try to give the quinine and clindamycin simultaneously rather than sequentially. As a preliminary study to establish the safety of simultaneous administration of these two drugs, 3 uninfected volunteers received successive courses of 3 days of quinine alone, clindamycin alone, and quinine and clindamycin simultaneously, according to the above dosage schedules. Blood levels of each drug were unaffected by simultaneous administration of the other, and there was no added toxicity from the drug combinations compared to each drug alone. Side effects consisted of gastrointestinal symptoms associated with either drug alone and there was no evidence of liver, renal or cardiac toxicity from the drugs.

Significance to Bio-medical Research and the Program of the Institute:

The widespread occurrence of multi-drug resistant Plasmodium falciparum in recent years has stimulated interest in the search for additional chemotherapeutic methods and drugs against malaria. Our studies thus far have shown that clindamycin, in combination with quinine, produced radical cure when tested against multi-drug resistant P. falciparum from Asia and Central America. Clindamycin appears to have an advantage over other antibiotics tested to date (tetracycline and minocycline) in that clindamycin may require a shorter course of therapy and has lower toxicity.

Proposed Course of the Project: In order to further streamline the quinine-clindamycin regimen, it is planned to attempt cure of P. falciparum infections with a schedule of 3 days of quinine (650 mg orally every 8 hours) and clindamycin (450 mg orally every 6 hours) simultaneously. This would reduce the treatment period from 6 days to 3 days. Five volunteers infected with the Burma (Thau) strain of P. falciparum will receive this regimen during the primary patency of their infections.

Serial No. NIAID 134-B
1. Parasitic Diseases
2. Human Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Characterization of New Isolates of Human Malaria

Previous Serial Number: Same

Principal Investigators: Louis H. Miller, Richard H. Glew and David J. Wyler

Other Investigators: Franklin A. Neva and William E. Collins

Cooperating Units: Department of Justice, Bureau of Prisons

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 34/12 | | | 34/12 |
| Professional | 4/12 | | | 4/12 |
| Other | 30/12 | | | 30/12 |

Project Description:

Objectives: To study the response to drugs of new isolates of P. falciparum. The new human malarial isolates will also be studied in the night monkey (Aotus trivirgatus).

Methods Employed: Healthy inmate volunteers of the U.S. Penitentiary, Atlanta, Georgia, are fully informed of the risks and discomforts of malaria, and are not subjected to coercion or excessive inducements. Volunteers are screened for mental and physical illnesses before they are admitted to the project, and are under the 24-hour care and supervision of the resident investigators. Volunteers are infected by exposure to the bites of several mosquitoes previously fed on infected Aotus trivirgatus monkeys. Participation in any one project may be for 6 to 18 months. Standard antimalarial regimens are administered and the patient observed for immediate clinical and parasitologic response.

Major Findings: 1. P. falciparum (Panama II strain): In March 1972 blood from a 10 year old boy from Rio Piedra on the Atlantic coast of the Republic of Panama was shipped to Chamblee, Georgia, where it was inoculated into Aotus trivirgatus monkeys. Anopheles freeborni mosquitoes fed on a single infected Aotus trivirgatus griseimembra monkey were used to infect 8 volunteers. Studies in the non-immune volunteers demonstrated that this Panama II strain of P. falciparum is resistant to several antimalarials including the 4-aminoquinolines (RI or RII to chloroquine, RI to amodiaquine) and the antifolates (RI to chlorquanide, RIII to pyrimethamine); in addition, treatment with quinine for 7 days was not curative (RI resistance). Infections with this strain were cured by treatment with 3 regimens: (1) quinine alone for 10 days (16.2gm, base, total); (2) combination of sulfadiazine for 5 days (10 gm total) with pyrimethamine for 3 days (150 mg total); (3) combination of quinine for 14 days (22.68 gm, base), sulfadiazine for 5 days and pyrimethamine for 3 days.

2. Central American strains of P. vivax: In July 1972 one of the volunteers previously infected with P. vivax (Salvador I strain) relapsed unexpectedly after presumably radical curative therapy with chloroquine and primaquine. This volunteer had been treated with chloroquine, 1.5 gm, base, and primaquine, 15 mg base daily for 14 days, in May 1971, six weeks after his first relapse. After transfer to the Ohio State Correctional Institution in Chillicothe, Ohio, the patient had a documented relapse of his P. vivax; treatment with quinine for 7 days followed by primaquine, 15 mg base daily for 14 days apparently was curative since the patient's infection has not relapsed since. The relatively long interval before relapse (greater than one year) in this patient may be explained by the fact that in this interval the volunteer had been infected with P. falciparum and received two courses of schizontocidal drugs for this latter infection; such therapy could have obscured possible P. vivax relapses during this time.

In April, 1973, another volunteer infected with a Central American vivax (Salvador II strain) relapsed one month after receiving chloroquine 1.5 gm, base, together with primaquine, 15 mg, base, daily for 14 days shortly following his first relapse. Of the other 49 volunteers infected by this Unit with Central American P. vivax in the past 5 years, none have been noted to relapse following 14 days of primaquine therapy.

Significance to Bio-medical Research and the Program of the Institute:

Failure of chloroquine to cure infections with P. falciparum was first reported in 1961. Since then, resistance of P. falciparum to 4-aminoquinolines and other groups of antimalarials has been confirmed in several regions of Southeast Asia, as well as in many areas of South America. However, it was not until 1972 that hospital studies of infections in Panamanian nationals documented resistance to 4-aminoquinolines in P. falciparum infections acquired north of Colombia, South America. Our studies in non-immune volunteers infected with a recently isolated strain of P. falciparum from Panama demonstrated resistance of this strain to a

variety of antimalarials, thus confirming the spread of multi-drug resistance to Central America.

Relapse of two infections with Central American P. vivax (Salvador I and Salvador Ii strains) after treatment with 14 days of primaquine serves as a warning of the unlikelihood of obtaining acceptable cure rates with significantly abbreviated courses of primaquine in the field.

Proposed Course of the Project: Studies of life patterns of P. vivax isolate and of drug responsiveness of P. falciparum isolates have been terminated.

Publications:

Contacos, P.G., Collins, W.E., Jeffery, G.M., Krotoski, W.A., and Howard, W.A.: Studies on characterization of P. vivax strains from Central America. Am. J. Trop. Med. & Hyg., 21: 707-712, 1972.

Collins, W.E., Contacos, P.G., Krotoski, W.A., and Howard, W.A.: Transmission of four Central American strains of P. vivax from monkey to man. J. Parasit., 58: 332-335, 1972.

Howard, W.A., Krotoski, W.A., Slonim, M.S., and Contacos, P.G.: Spontaneous splenic rupture in vivax malaria: Case Report, Milit. Med., 131: 31-35, 1973.

Glew, R.H., and Howard, W.A.: Transverse furrows of the nails associated with Plasmodium vivax malaria. Johns Hopkins Med. J., 132: 61-64, 1973.

Serial No. NIAID 134-C
1. Parasitic Diseases
2. Primate Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

**Project Title:** Cardiopulmonary Changes in Malaria in Human Volunteers

**Previous Serial Number:** Same

**Principal Investigators:** Richard H. Glew and Louis H. Miller

**Other Investigators:** None

**Cooperating Units:** Department of Justice, Bureau of Prisons

**Man Years:**

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 2/12 | | | 2/12 |
| Professional | 2/12 | | | 2/12 |
| Other | | | | |

**Project Description:**

Objectives: This study was designed to evaluate possible changes in arterial oxygen saturation during the malarial paroxysm.

Methods Employed: Volunteers infected for other purposes with P. vivax and P. falciparum by the bites of infected mosquitoes were studied. Routine spirometry was performed prior to study. In each volunteer, one or two arterial blood samples were drawn during the paroxysm under study, during either the chilling or flush phases. Control arterial blood gas studies were obtained after therapy when the volunteer was afebrile, asymptomatic and smear negative for malaria. Physical examinations, chest x-rays, and electrocardiograms were made serially during the study.

Major Findings: Nine arterial blood samples were drawn during malarial paroxysms in seven volunteers (six with P. falciparum and one with P. vivax). All of the samples were obtained while the patients were febrile (temperatures

between 38.4 and 40.0°C); only one sample was drawn during a true shaking chill; four samples were drawn during mild chills which occurred while the volunteers were already febrile in a flush state of peak fever. There were no significant changes in arterial $pO_2$, $pCO_2$ or pH as compared with control samples. In addition, no changes were noted in serial physical examinations, electrocardiograms, or chest x-rays.

Significance to Bio-medical Research and the Program of the Institute:

Respiratory complaints without obvious etiology have been described in the older malaria literature. Furthermore, it has been observed by this Unit that occasional human volunteers experimentally infected with malaria have complaints of mild, transient dyspnea and cough during paroxysms. However, arterial blood gas studies performed in seven volunteers infected with malaria were all normal. Therefore, these studies at low levels of parasitemia offer little information on the pathophysiology of severe malaria.

Proposed Course of the Project: This project is completed.

Serial No. NIAID 134-D
1. Parasitic Diseases
2. Human and Primate Malaria
3. Atlanta and Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Limulus Assay for Endotoxin in Experimental Malaria

Previous Serial Number: None

Principal Investigator: Richard H. Glew

Other Investigators: None

Cooperating Units: Jack Levin, Department of Medicine, Johns Hopkins Hospital; Department of Justice, Bureau of Prisons

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Worker | Total |
|---|---|---|---|---|
| Total | 1/12 | | | 1/12 |
| Professional | 1/12 | | | 1/12 |
| Other | | | | |

Project Description:

Objectives: The possibility that an endotoxin-like substance plays a role in malaria is suggested by the clinical similarity between human malaria and the pyrogen reaction to endotoxin, as well as by the occurrence of endotoxin tolerance in humans infected with malaria. The purpose of this study is to attempt to demonstrate endotoxin in the plasma of patients and monkeys infected with malaria.

Methods Employed: Human volunteers, previously infected with P. vivax and P. falciparum for other purposes by the bites of infected mosquitoes were studied. In general, plasma samples were obtained in well-synchronized infections; serial plasma samples were drawn beginning in the afebrile, asymptomatic period prior to a paroxysm, as well as on one or more occasions during the chilling and febrile periods of a paroxysm. In order to study infections during high parasitemia, two monkeys infected with P. coatneyi were also studied, with samples drawn just prior to, and during schizogony.

Major Findings: All plasma samples failed to cause gelation of the amebocyte lysates from Limulus, and therefore are considered negative for endotoxin (This assay is capable of detecting endotoxin in concentrations as low as 0.0005 μg/ml). This survey consisted of 15 samples from four volunteers infected with P. vivax, six samples from two volunteers infected with P. falciparum, and nine samples from two rhesus monkeys infected with P. coatneyi.

Significance to Bio-medical Research and the Program of the Institute:

This study was begun as part of a continuing investigation into the etiology and pathophysiology of fever and of malaria in general. Our inability to demonstrate endotoxin in the plasma of humans or monkeys infected with malaria suggests that a substance other than endotoxin probably is responsible for the malarial paroxysm.

Proposed Course of the Project: This series of negative investigations is felt sufficient to preclude the need for further attempts at demonstrating endotoxin in malaria.

Serial No. NIAID 67 C

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on arboviruses other than dengue

Previous Serial Number: Same

Principal Investigator: Dr. Robert Tesh

Other Investigators: Dr. Leon Rosen, Dr. Duane Gubler

Cooperating Units: Department of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine, Honolulu; Middle America Research Unit, Ancon, Canal Zone; Yale Arbovirus Research Unit, Yale University School of Medicine, New Haven, Conn.

Man Years:

| | NIAID staff |
|---|---|
| Total: | 6/12 |
| Professional: | 6/12 |
| Other: | 0 |

Project Description:

Objectives

1. To study the epidemiology of arthropod-borne virus infections other than dengue of public health importance on the Pacific islands and in Southeast Asia.

2. To study the genetics of susceptibility of mosquitoes to Chikungunya and yellow fever viruses as compared with that of dengue viruses in laboratory colonies of Aedes albopictus.

3. To study the role of arthropod-borne viruses other than dengue as etiologic agents of hemorrhagic disease.

## Methods Employed

Studies of experimental arbovirus infection of mosquitoes after intrathoracic inoculation or feeding were done utilizing a laboratory colony of Aedes albopictus. Titration of infected mosquito specimens was done by tube culture or plaque production in LLC-$MK_2$ and Vero cells. Plaque neutralization studies of human sera were performed in microplate cultures of LLC-$MK_2$ cells.

## Major Findings

### Development of Chikungunya virus in mosquitoes

Experiments were initiated to study the development of Chikungunya virus in A. albopictus infected by ingestion. Preliminary data indicate that at 28°C, after an initial lag phase, the virus reaches maximum titers about the seventh or eighth day following ingestion. In comparing the sensitivity of various culture systems for detecting Chikungunya infection it was found that titration of virus specimens in mosquitoes (A. albopictus) by intrathoracic inoculation was 10 to 1000 times more sensitive than LLC-$MK_2$ tube or microplate cultures.

### Neutralization studies on sera from Chikungunya patients

Hemagglutination-inhibition studies of human sera from the Pacific islands have shown varying degrees of cross-reaction to several Group A arbovirus antigens, making interpretation of results and identification of the specific infecting agent difficult. In hopes of finding a more specific test, plaque neutralization studies were performed on acute and convalescent sera from Asian patients hospitalized with Chikungunya disease. Preliminary data indicate that persons infected with Chikungunya develop high titers of specific neutralizing antibodies which do not cross-react with three other Group A arboviruses from the same geographic area.

### Experimental infection of mosquitoes with the 17D vaccine strain of yellow fever virus

It was demonstrated that the 17D strain of yellow fever virus replicates in A. albopictus following intrathoracic inoculation. This indicates that despite over 200 passages in mice and in chick embryo culture, the virus has retained its infectivity for mosquitoes. Studies are in progress to determine if A. albopictus can be infected by ingestion of virus and whether infected mosquitoes can transmit the 17D strain by bite to susceptible animals.

Because of the hazards of working with virulent yellow fever virus, studies of its development (growth curve, maximum titers, etc.) in mosquitoes have not been performed. In view of recent outbreaks of yellow fever in Africa and of the reintroduction of Aedes aegypti, the urban vector, into Brazil, Panama and Costa Rica, further studies of the virus-vector relationship would seem desirable. The finding that the attenuated 17D vaccine strain of yellow fever virus grows in mosquitoes may provide a safe model for such laboratory studies.

Most of the work on sandfly (papataci) fever has been done in the Mediterranean region where the disease has long been recognized as a public health problem. However, viruses belonging to the Phlebotomus fever group recently have been isolated in Africa, India, the Middle East and the Americas, thus extending the known geographic distribution of these agents. Recovery of two of the newer members of the group from sick humans in Panama suggests that sandfly fever is not restricted to arid areas of the Old World. Despite numerous published clinical descriptions of sandfly fever, little is known about the epidemiology of the disease or of the true prevalence of human infection. Repeated recoveries of Phlebotomus fever group viruses from pools of naturally infected male sandflies plus recent demonstration of transovarial transmission of vesicular stomatitis virus in these insects suggests that transovarial transmission of certain sandfly viruses may occur in nature and that the ecology of these agents may be quite different from mosquito-borne viruses. Studies on the Phlebotomus fever group of arboviruses are designed to learn more about their ecology, serologic relationships, and geographic distribution as well as the prevalence of human infection with these agents.

Proposed Course of the Project

Serologic surveys by plaque neutralization test are planned on human sera from various Pacific Islands for evidence of Group A arbovirus infection. Because of the limited fauna of these islands, if a focus of virus activity can be identified, it would provide an ideal locality for further study of the epidemiology of disease produced by this group of agents.

From information gained in studies of the development of Chikungunya virus in A. albopictus, we plan to attempt through genetic selection and inbreeding to develop resistant and susceptible laboratory mosquito strains. If successful, this model should provide valuable new information on the genetic factors controlling mosquito susceptibility to arbovirus infections. Studies will be done to determine whether Chikungunya-resistant strains are also resistant to dengue, and vice versa, or whether genetic resistance to arboviruses is only group or type specific.

Inoculation of mosquitoes with sandfly-associated viruses

Adult female A. albopictus were inoculated intrathoracically with Punta Toro, Icoraci, Sicilian and Changuinola viruses. Punta Toro, Icoraci and Sicilian were detected in mosquitoes immediately post-inoculation but not in specimens tested on days 7 and 14 after inoculation. Punta Toro and Sicilian viruses were also inoculated into a laboratory strain of Culex fatigans. The results were the same as with A. albopictus. In contrast, Changuinola virus was present in all mosquitoes tested (immediately post-inoculation and on days 7 and 14), indicating persistence or multiplication in A. albopictus. These data are similar to those with insect cell culture, which indicate that Phlebotomus fever group arboviruses do not multiply in mosquito cells. Changuinola virus, on the other hand, is an ungrouped arbovirus which, on the basis of physico-chemical properties and morphology, appears to belong to the Diplornovirus group.

Serologic studies of the Phlebotomus fever group

These studies are a continuation of those begun by the principal investigator at the Middle America Research Unit. At present, a total of 21 distinct viral agents from various regions of the world are included in the Phlebotomus fever arbovirus group on the basis of serologic relationships demonstrated by hemagglutination-inhibition tests. A number of these agents have never been described and a complete serologic study of the entire group has not been done. Cross-neutralization and complement fixation tests with specific hyperimmune hamster sera prepared against each of the 21 viruses have shown little cross-reaction. Hemagglutination-inhibition tests with the same sera are in progress at the Yale Arbovirus Unit.

Significance to Biomedical Research and Program of the Institute

Chikungunya, a group A arbovirus, has been responsible for major epidemics of dengue-like illness among human populations in Africa, India, the Philippines, and several countries of Southeast Asia. Since dengue and Chikungunya infection are similar clinically, and as both agents may occur simultaneously in the same area, differentiation of illness produced by the two viruses is of obvious public health importance. Like dengue, Aedes aegypti has been implicated as the principal urban vector during epidemics of Chikungunya disease. However, despite a common vector, overlapping geographic distribution, and clinical similarity with dengue, isolations of Chikungunya virus from other mosquito genera (Anopheles, Culex and Mansonia) as well as its occurrence in dengue-free areas of Africa suggest that the ecology of the two viruses is different. These studies are designed to learn more about the epidemiology of Chikungunya disease.

Further studies are also planned on the development of the 17D strain of yellow fever virus in A. albopictus and eventually in A. aegypti. The susceptibility of mosquitoes to the 17D strain may provide a useful model for biological studies of the development of yellow fever virus in vector species without the hazards of working with a virulent virus strain. In addition, 17D will be used to test the susceptibility of dengue-resistant mosquito strains to another important Group B arbovirus.

Plans are also being made to develop collaborative programs with several laboratories in Southeast Asia to study the prevalence of sandfly associated viruses in this region. Although sandfly fever has been reported in India, China and Eastern Russia, nothing is known about the distribution of sandfly viruses or prevalence of human infection in Southeast Asia.

**Honors and Awards:**

None

**Publications:**

Tesh, R.B., and Johnson, K.M.: Vesicular stomatitis. In Hubbert, W.T., McCulloch, W.F. and Schnurrenberger, P.R. (eds.): Diseases Transmitted from Animals to Man, Sixth Edition. Springfield, Illinois, Charles C. Thomas, 1973 (in press).

Tesh, R.B., Ackerman, L.J., Dietz, W.H. and Williams, J.A.: Angiostrongylus costaricensis in Panama. Prevalence and pathologic findings in wild rodents infected with the parasite. Amer. J. Trop. Med. Hyg., 1973 (in press).

Martin, D.H., Chaniotis, B.N. and Tesh, R.B.: Host preference of Deinocerites pseudes Dyar & Knab. J. Med. Entomol., 1973 (in press).

Serial No. NIAID 67D
1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the natural history of Toxoplasma gondii, the mechanism of its transmission to man, and its public health importance in the Pacific area.

Previous Serial Number: Same

Principal Investigator: Dr. Gordon D. Wallace

Other Investigators: Dr. D. Carleton Gajdusek, Dr. Robert Moellering, Dr. Alistair Philip, Dr. Vincent Zigas

Cooperating Units: Study of Child Growth and Development and Disease Patterns in Primitive Cultures, NINDS; Infectious Disease Unit, Department of Medicine, Harvard Medical School; Department of Pediatrics, University of Hawaii School of Medicine; Public Health Department of the Territory of Papua and New Guinea; Hawaii State Department of Public Health.

Man Years: NIAID staff

| | |
|---|---|
| Total: | 5/12 |
| Professional: | 5/12 |
| Other: | 0/12 |

Project Description:

Objectives

To determine the method, or methods, by which man becomes infected with T. gondii and the public health importance of this parasite, particularly in undeveloped areas with a high prevalence of human infection.

As a related project, limited serologic studies were done to determine if man is naturally infected with another coccidia of cats, Isospora felis.

## Methods Employed

### Toxoplasma

The role of the cat in the life history of Toxoplasma and its transmission to man was studied on certain Pacific Islands. The island of New Guinea provided a particularly interesting ecologic situation because felids are not indigenous there and the distribution of the introduced domestic cat is still limited. There are vast regions inhabited by native New Guineans where cats have never been present, compared to other inhabited locations where cats have been in the environment for various periods of time. The prevalence of Toxoplasma dye-test antibody in selected populations of New Guineans was compared with the estimated probability of their contact with cats.

In another study designed to determine if Toxoplasma was maintained in nature in the absence of cats, rats on small Pacific Islands, uninhabited by man or felids, were tested for Toxoplasma antibody. These results were compared with serologic data from rats tested on islands where cats were present.

Acquired toxoplasmosis and chorioretinitis. To test the theory that acquired toxoplasmosis seldom causes eye disease, serum specimens from populations of natives on two remote Solomon Islands which had been examined ophthalmologically in a study of eye disease in general, were tested for Toxoplasma antibody. It was suspected that these populations would have a very high prevalence of infection with Toxoplasma.

Congenital toxoplasmosis. To evaluate the public health importance of congenital toxoplasmosis in Hawaii and to possibly provide an opportunity for long term observation of babies born with subclinical toxoplasmosis, a prospective study of newborns in Hawaii was undertaken. As a pilot study, sera from umbilical cords of 970 babies were screened for dye-test antibody at a 1:512 dilution. Additional specimens were collected and tested at appropriate times from babies with positive cord serum.

### Isospora felis

A serologic test for antibody to I. felis was developed utilizing the indirect fluorescent antibody technique, and sporozoites excysted from oocysts were used as antigen. Human sera from the following groups were tested: 1) 113 young adult Costa Ricans with a high prevalence of Toxoplasma antibody, living under conditions in which exposure to cats and cat feces was likely; 2) 25 veterinarians or veterinary assistants in Honolulu, 3) seven employees of the Honolulu Humane Society. Human and mouse sera with low, medium, and high Toxoplasma antibody titers were also tested.

## Major Findings

### Toxoplasma

The role of the cat. Sera from more than 1000 primitive New Guineans, including nine different cultural-linguistic groups inhabiting widely separated geographic areas, were tested for Toxoplasma antibody. Toxoplasma antibody was not detected, or was found in less than two percent of New Guineans living in areas where felids had never been present, or where they had been recently introduced and were still limited in number and distribution. However Toxoplasma antibody was detected in 14 to 34 percent of New Guineans living in areas where cats were more numerous and had been present longer.

Sera from 900 rats on four small Pacific Islands, uninhabited by humans or felids were tested for Toxoplasma antibody, and none were positive. In comparison, tests on sera from 878 rats on three small islands where cats had been present revealed the presence of Toxoplasma antibody in 1 to 12 percent.

Acquired toxoplasmosis and chorioretinitis. An ophthalmologic survey that included examination of the fundi of almost the entire populations of Ontong Java (about 600 people) and Ulawa (about 300 people), both remote islands in the Solomon group, revealed only one case of chorioretinopathy in each population. The prevalence of Toxoplasma antibody on Ontong Java was 90% and on Ulawa it was 50%. It was apparent that congenital toxoplasmosis would have been a rare event on Ontong Java since most females on the island had acquired antibody by the time they had reached child bearing age. Although there are no available statistics on the prevalence of chorioretinopathy in other comparable populations, it was apparent that acquired toxoplasmosis in the populations studied was causing little or no eye disease.

Congenital infection. Of cord sera tested from 970 babies born on Oahu, Hawaii during a four-month period, 48 (5%) were found to have dye-test antibody titers of $\geq$ 1:512. Thirteen had titers of $\geq$ 1:2,048. Antibody titers had decreased four-fold or greater in all but six of 40 babies from whom at least one follow up specimen was obtained (one to eight months after birth). It was possible to obtain additional specimens from four of the six babies whose titers had remained elevated, and a significant decrease in titer had occurred in all but one. This baby was subsequently found to have active toxoplasmosis that resulted in severe loss of vision and possible brain damage. When the data from the study were analyzed by ethnic backgrounds of the babies, it was found that 13 (28%) of the 48 babies with antibody titers in cord blood of $\geq$ 1:512 and 8 (62%) of the 13 babies with titers of $\geq$ 1:2,048 were Filipinos. Only about 10% of all of the 970 babies tested, however, were Filipinos. In addition, three of five confirmed clinical cases of congenital toxoplasmosis recognized during the past 18 months in Hawaii (including the one in the study) were Filipinos. Three other presumptive cases were also in Filipino children. Because of this information, it has been decided to limit the study to Filipino babies.

I. felis

Human sera were screened at a dilution of 1:32 because non specific fluorescent staining of antigen occurred frequently at lower serum dilutions. Of the 113 Costa Rican sera tested, six had titers of 1:32 to 1:64. All of the sera from veterinarians and Humane Society employees were negative at 1:32. Of interest was the finding that both human and mouse sera with high dye-test titers (human $\geq$ 1:32,000, mouse $\geq$ 1:2,000) had low I. felis titers (1:16 - 1:64). None of the I. felis positive Costa Rican sera, however, had high Toxoplasma antibody titers.

## Significance to Biomedical Research and the Program of the Institute

### Toxoplasma

Human toxoplasmosis is recognized as a medical problem throughout the world and until recently information on the life history of Toxoplasma has been insufficient to offer much hope for prevention and control. Now that the role of the cat has been more clearly defined, it is possible to recommend certain preventive measures, which at least in developed countries have practical application. The prevalence of human infection with Toxoplasma in undeveloped tropical countries, however, is relatively high and more information on the public health importance and specific modes of human infection (cats, raw meat, cockroaches) are needed. Although current information suggests that toxoplasmosis is a minor problem in populations with high prevalences of antibody, because most females acquire immunity before reaching child bearing age, Toxoplasma may cause serious health problems when introduced into previously unexposed populations, such as in New Guinea. The incidence of serious human disease from the parasite may also increase in tropical countries undergoing economic development because improved living standards and sanitation may reduce the overall incidence of human infection resulting in more susceptibles of child bearing age being at risk.

### I. felis

Since it is now generally accepted that a significant number of human infections with Toxoplasma are the result of exposure to Toxoplasma oocysts from cat feces, it would be of interest to determine if man similarly would become infected with the oocyst of I. felis, a coccidia much more common in cats than is Toxoplasma. The results to date, although not confirmed, suggest that human infection with I. felis is infrequent and insignificant.

## Proposed Course of the Project

### Toxoplasma

The project on congenital toxoplasmosis will be continued to determine if enough subclinical cases can be found to provide the potential to study the long term residual effects of inapparent congenital infection, a subject about which little is known. Serious central nervous system damage may eventually occur in such children.

A study on the epidemiology of toxoplasmosis in Taiwan is being formulated in collaboration with U.S. Naval Medical Research Unit No. 2. Preliminary information indicates that human infection in that country is very low while the prevalence of the parasite in lower animals may be as high as that on other Pacific Islands (where the prevalence of human infection is high). If this can be confirmed, it would be of interest to determine why man is so infrequently infected in Taiwan in relation to other Pacific island populations.

### I. felis

It would be interest to test human sera with antigen of tissue stages of I. felis, such as merozoites and trophozoites. However at present this is not planned because of the technical difficulties involved. Since children might be expected to have been infected more recently than adults and have higher titers, sera from a group of Costa Rican children will be tested. If the results of these tests are essentially negative, the project will be terminated.

### Honors and Awards:

Dr. Wallace continued to serve as Assistant Clinical Professor of Public Health and Assistant Clinical Professor of Tropical Medicine and Medical Microbiology in the University of Hawaii Schools of Public Health and Medicine, respectively.

### Publications:

Wallace, G.D., Marshall, L., Marshall, M.: Cats, rats, and toxoplasmosis on a small Pacific island. Amer. J. Epidem., 95: 475-482, 1972.

Wallace, G.D.: Experimental transmission of Toxoplasma gondii by cockroaches. J. Infect. Dis., 126: 545-547, 1972.

Wallace, G.D.: The role of the cat in the natural history of Toxoplasma gondii. Am. J. Trop. Med. Hyg., 22: 313-322, 1973

Wallace, G.D.: Intermediate and transport hosts in the natural history of Toxoplasma gondii. Am. J. Trop. Med. Hyg., 1973 (in press).

Serial No. NIAID 67H

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on dengue

Previous Serial Number: None

Principal Investigator: Dr. Leon Rosen

Other Investigators: Dr. Duane Gubler, Dr. Timothy Kuberski, Dr. Lloyd Rozeboom, Dr. William Scherer, Dr. Peter Beales, Dr. Winton Barnes

Cooperating Units: School of Hygiene and Public Health, Johns Hopkins University, Baltimore; Cornell University Medical College; Government of American Samoa; Government of Niue, Niue Island.

Man Years:

| | NIAID Staff | Guest Worker |
|---|---|---|
| Total: | 24/12 | 6/12 |
| Professional: | 24/12 | 6/12 |
| Other: | 0 | 0 |

Project Description:

Objectives

1. To study the epidemiology of dengue and dengue hemorrhagic fever in the simple ecologic environment of Pacific Islands.

2. To study the relative susceptibility of various species of mosquitoes to infection with dengue viruses.

3. To improve laboratory methods for the detection of dengue viruses and their antibodies.

Methods Employed

Epidemics of dengue are studied as they occur on Pacific islands. During the year, investigations were carried out on two outbreaks of dengue, one in American Samoa and the other on Niue Island.

Also, various species of mosquitoes, Macaca monkeys, and chimpanzees were infected experimentally with strains of dengue virus (including the four prototypes) which had not been potentially modified by previous passage in mice or cell cultures.

Various virologic techniques previously developed for use with non-arthropod borne viruses were used in an attempt to improve the techniques currently available for work with the dengue viruses.

Major Findings

American Samoa dengue outbreak

Dengue was diagnosed in American Samoa in May, 1972 after an absence of more than 25 years. Field investigations were undertaken with two principal objectives in mind, 1) to compare levels of viremia and clinical manifestations in persons undergoing primary as compared to secondary dengue infections, and 2) to evaluate the role of Aedes species other than aegypti in the transmission of the disease. It was known from a previous serologic survey that almost everyone who lived in American Samoa during World War II had acquired dengue and hence would be at risk of experiencing a secondary dengue infection in the new outbreak. Conversely, anyone born since that time would experience a primary infection. Furthermore, it was known that A. aegypti could not be found in American Samoa in 1965 and that only a small focus of this species had been found in 1968.

In view of the magnitude of the outbreaks which occurred on Tahiti and New Caledonia during the previous year it was anticipated that the outbreak on American Samoa would be of similar proportions. This was not the case - probably because of limited A. aegypti population in American Samoa as compared with that in the other two localities. Although it was not possible to feed Aedes polynesiensis on as many patients as had been hoped, the sum of the epidemiologic evidence was that the outbreak was transmitted exclusively by Aedes aegypti despite an abundant Aedes polynesiensis population.

The number of patients observed by the staff of the Section with proven dengue infections was too small (approximately 30) to make valid clinical comparisons. However, dengue virus (type 2) was isolated from 18 individuals and these data on viremia along with similar data from the Tahiti and New Caledonia outbreaks should furnish a fairly comprehensive picture of the level and duration of viremia in these type 2 outbreaks.

One unanticipated dividend from the investigation of the American Samoa outbreak was the opportunity to study the decay in dengue HI and neutralizing antibody over a period of approximately 8 months. These data proved valuable in interpreting serologic data from the Niue Island dengue outbreak (see below).

Niue Island dengue outbreak

An outbreak of dengue associated with an unusually high incidence of minor hemorrhagic phenomena and 12 deaths - including some in young children which were clinically suggestive of the dengue shock syndrome - occured on relatively remote and sparsely populated (4600 people) Niue Island during the first part of 1972. This was a surprising event since it is generally believed that severe and fatal forms of dengue occur only in areas where two or more dengue serotypes are endemic or occur sequentially within a relatively short period of time (i.e. 5 years). Previously acquired data on the past and current distribution of dengue viruses on Pacific islands indicated that it was unlikely that the above conditions had prevailed on Niue Island.

It was not possible to investigate the Niue outbreak while it was in progress, but a retrospective investigation was undertaken in hope of determining whether or not more than one dengue virus had been present during the outbreak, or in the recent past, and whether or not the associated deaths were likely to have been caused by dengue virus. With the aid of a newly developed plaque-reduction neutralization test, it was demonstrated that dengue type 2 virus had caused the recent outbreak and that no other type of dengue virus had been present on the island during the previous 28 years. Most persons living on the island prior to 1944 had acquired dengue type 1 infection (as had those living on many other Pacific islands at that time). An analysis of hospital records and interviews with the families of severe and fatal cases indicated that the severe and fatal disease which had occurred on Niue was typical of that described previously in association with dengue infections in Southeast Asia.

Comparative susceptibility of different mosquito species to dengue

The testing of various species of mosquitoes for susceptibility by ingestion to various types and strains of dengue virus was continued with exposure of Stegomyia species and Culex and Armigeres to dengue type 4 and to several strains of dengue type 3. As was found previously with dengue types 1 and 2, all of the Stegomyia species became infected whereas neither Culex nor Armigeres did so. Again, as with dengue types 1 and 2, a higher percentage of apparently resistant individuals was found in the case of Aedes aegypti than other Stegomyias exposed to dengue type 4. In the case of dengue type 3, however, Aedes polynesiensis appeared to be by far the most resistant species. This phenomenon, observed with the prototype strain,

was confirmed with two other dengue type 3 strains (from Thailand and the Philippine Islands) and in the case of one of these virus strains, with two different colonies of Aedes polynesiensis (from Samoa and Tahiti). The colony of A. aegypti that was relatively resistant to dengue types 1, 2, and 4 also appeared more resistant to one dengue 3 strain than did Aedes albopictus, but another colony of A. aegypti did not.

Because of continued difficulty in propagating a strain of dengue 3 from Tahiti in A. albopictus by injection, various other Stegomyias were employed. It was found that this dengue strain grew to higher titer in A. polynesiensis than it did in A. albopictus. With the one exception noted in the annual report for last year (dengue 3 in Aedes scutellaris), this is the only instance in which it has been found that the Stegomyias vary in their susceptibility to, or ability to propagate, dengue viruses after intrathoracic inoculation.

Dengue viremia studies in Macaca monkeys and chimpanzees

The magnitude and duration of viremia following infection by mosquito bite was studied in Macaca irus monkeys undergoing primary infections with dengue types 1, 2, and 4. It was found that although the duration of the viremias was roughly similar to that which occurs in man, their magnitude was considerably lower (10 to 100 fold). Despite these lower levels of viremia, some A. albopictus became infected after feeding. This same mosquito species failed to become infected when ingesting similar amounts of virus in artificial feeding mixtures, but did do so when they ingested much larger amounts of virus in such mixtures.

The magnitude and duration of viremia following subcutaneous inoculation of unmodified dengue viruses were also studied in chimpanzees infected with each of the four dengue serotypes (two animals with each type). The duration of the viremias was similar to that observed in the monkeys but their magnitude appeared to be somewhat higher. No mosquitoes were fed on these animals (which were housed in New York). Some of the animals had had naturally acquired Group B arbovirus infections (not dengue) prior to their experimental dengue infection.

Improvement in dengue laboratory techniques

The production of CF antigen in mosquitoes inoculated intrathoracically was studied further and typing technique perfected to the point that it was possible to identify all available dengue isolates by this method. More than 100 strains including representatives of all four serotypes were employed in these studies. The CF antigen could be detected in a single mosquito about 50 percent of the time. When a pool of 5 infected insects was employed, identification was possible in every instance.

Although the use of mosquitoes to carry out neutralization tests was not successful, a plaque-reduction neutralization technique employing multi-welled plastic plates was perfected for all four serotypes and used extensively in studying sera from the Niue Island outbreak. Apparently, a small fraction of dengue virus is resistant to neutralization, and because of the sensitivity of mosquitoes to infection, this virus replicates rapidly. On the other hand, the plaque-reduction neutralization technique avoids this problem because the presence of a relatively few unneutralized plaques does not affect the interpretation of the test.

Extensive work was carried out to perfect the detection of mosquitoes infected with dengue by the fluorescent antibody technique. The indirect method did not prove successful, but it now appears that it will be possible to develop the direct method to the point where infected mosquitoes can be identified on a routine basis. This technique was also used successfully to follow sequentially the sites of virus development in the mosquito.

Significance to Biomedical Research and the Program of the Institute

Dengue viruses are increasing in significance as a cause of morbidity and mortality in Southeast Asia and Pacific islands - probably because of rapidly increasing urbanization and travel in that geographic area. Mosquito vectors of these viruses are ubiquitous in the same area, which includes many islands belonging to, or controlled by, the United States. Obviously, further understanding of the epidemiology of dengue and of the vectors of the disease would be of value.

The findings from Niue Island outbreak will have considerable impact on several aspects of dengue research. They indicate that fatal dengue could occur the first time a particular dengue serotype appeared in an area (including the Caribbean area and the Southern United States) - if it happened to be a virulent strain. As a result, the desirability of developing an effective vaccine is increased since, 1) it would be more likely to be needed and, 2) one need not fear the risk of dengue "sensitization".

If it proves possible to perfect the fluorescent antibody technique for the routine detection of mosquitoes infected with dengue, the laboratory tools available for the study of dengue viruses will be impressive; inoculation of male mosquitoes for the isolation of viruses, their titration, and the production of high-titered virus pools; fluorescent antibody technique for the detection of infected mosquitoes; and complement-fixation for type-specific identification of dengue isolates. None of these methods require either mouse colonies or cell culture facilities and hence place the possibility of sophisticated dengue research within the reach of the smallest laboratory.

Proposed Course of the Project

Further field studies on dengue outbreaks will be undertaken as appropriate opportunities occur. An attempt will also be made to write up for publication the extensive amount of data already on hand from outbreaks investigated previously.

Work on the comparative susceptibility of various species of Stegomyias to different dengue strains will be continued and expanded in collaboration with Dr. Byron Chaniotis at the Rocky Mountain Laboratory.

The quantitative studies of dengue viremia in the cynomolgus monkey will be continued with the study of animals undergoing secondary-type dengue infections.

Considerable effort will be devoted to perfecting the fluorescent antibody technique for the routine detection of infected mosquitoes.

Honors and Awards:

Dr. Rosen was appointed to the editorial board of the new journal, Intervirology. He continued to serve as a member of the Viral Diseases Panel, U.S.-Japan Cooperative Medical Science Program; as a member of the ad hoc Enterovirus Advisory Group and of the ad hoc Arbovirus Advisory Group, NIAID; on the Executive Committee of the American Committee on Arthropod-borne Viruses; as Clinical Professor of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine; and as a member of the Affiliate Graduate Faculty, University of Hawaii School of Public Health.

He also served as co-organizer and co-chairman of a workshop on "Factors Affecting the Competence of Diptera as Vectors of Arboviruses and Filariae" held under the joint sponsorship of the U.S. Viral and Parasitic Diseases Panels of the U.S.-Japan Cooperative Medical Science Program.

Publications:

Moreau, J-P, Rosen, L., Saugrain, J., and Lagraulet, J.: An epidemic of dengue on Tahiti associated with hemorrhagic manifestations. Amer Jour Trop Med Hyg 22:237-241, 1973.

Rosen, L., Schmidt, N.J., and Kern, J.: Toluca-1 a newly recognized enterovirus. Arch Ges Virusforsch 40:132-136, 1973

Rozeboom, L.E., Rosen, L., and Ikeda, J.: Observations on oviposition of Aedes (S.) albopictus Skuse and A. (S.) polynesiensis Marks in nature. Jour. Med. Entomol. (in press)

Loison, G., Rosen, L., Papillaud, J., Tomasini, J., Vaujany, J., and Chanalet, G.: La dengue en Nouvelle-Caledonia (1971-1972). Bull. Soc. Pathol. Exot.

Rosen, L.: Reoviruses. In Hubbert, W.T., McCulloch, W.F. and Schnurrenberger, P.R. (eds.): Diseases Transmitted from Animals to Man, Sixth Ed. (in press)

Dean, A.G., Couch, R.B., Jones, T.C., and Douglas, Jr., R.G.: Seasonal gastroenteritis and malabsorption at an American military base in the Philippines. III. Microbiologic investigations and volunteer experiments. Amer. Jour. Epidemiol. 95:451-463, 1972.

Dean, A.G., Ching, Y.-C., Williams, R.G., and Harden, L.B.: Test for Escherichia coli enterotoxin using infant mice: Application in a study of diarrhea in children in Honolulu. Jour. Infect. Dis. 125:407-411, 1972.

Serial No. NIAID 67-I

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Susceptibility and Resistance of Mosquitoes to Dengue

Previous Serial Number: None

Principal Investigator: Dr. Duane J. Gubler

Other Investigators: Dr. Leon Rosen

Cooperating Units: Department of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine, Honolulu.

| Man Years: | NIAID Staff | Guest Worker |
|---|---|---|
| Total: | | 6/12 |
| Professional: | | 6/12 |
| Other: | | 0 |

Project Description:

Objectives

1. To select strains of A. albopictus which are either highly resistant or highly susceptible to infection with dengue viruses.

2. To study the genetic basis of resistance to infection.

3. To study replication and distribution of dengue virus in resistant and susceptible mosquitoes.

Methods Employed

Attempts to infect Aedes (S.) albopictus and other species of Stegomyia orally with dengue viruses usually result in rates of infection of less than 100 percent. Strains of A. albopictus from different geographic areas will be screened for susceptibility to infection with dengue viruses. Those strains showing the highest natural resistance will be selected for resistance

by feeding on suspensions with high virus concentration whereas selection pressure for increased susceptibility will be placed on those strains showing the highest natural susceptibility by feeding on suspensions with low virus concentration. Reciprocal and backcrossing experiments will be made to determine the type of inheritance controlling vectorial capacity after pure lines have been established.

Studies will be made on the replication and distribution of dengue virus in mosquitoes in relation to resistance and susceptibility. Methods employed for these studies will include virus titration of various mosquito organs and tissues by mosquito assay and determination of the site of virus replication by fluorescent antibody and, possibly, electron microscopy.

Major Findings

Preliminary feeding experiments suggest that different Stegomyia species vary in their susceptibility to dengue viruses and that a single mosquito species may exhibit differential susceptibility to several strains of the same serotype. In artificial feeding experiments, the amount of virus required to infect Hawaiian A. albopictus appeared to be higher for dengue 1 and 4 than for dengue 2. Preliminary data from mosquitoes fed on viremic monkeys support this conclusion. In comparing infection rates in mosquitoes fed on artificial suspensions and on monkeys it was found that the threshold of infection was considerably higher for the artificial feeding suspensions. Thus, mosquitoes feeding on monkeys became infected after ingesting only 10 to 50 $MID_{50}$ whereas few mosquitoes which fed on a hanging drop suspension were infected after ingesting 500 to 1000 $MID_{50}$.

Selection for resistance has been started using individual mosquitoes which were naturally resistant to oral infection; individuals which fed on the same virus suspension, but were infected, are being selected for increased susceptibility. The results of these preliminary experiments are not yet available.

Significance to Biomedical Research and the Program of the Institute

Dengue viruses are widespread in Asia and the Pacific and outbreaks of the disease associated with severe hemorrhage or shock make dengue one of the most important public health problems of that area. Control of the vector is presently the only available means of controlling the disease. One possible method of control would be to replace a susceptible vector species with a refractory strain of the same species. Another possible approach would be to increase the resistance of a natural vector population by introducing the gene controlling this factor into the population. Theoretically, either of these methods, which depend upon selection of resistant mosquito strains in the laboratory, could interrupt transmission and bring about permanent disease control.

Proposed Course of the Project

Mosquito strains from different geographical locations will be screened for natural susceptibility and resistance. Selection experiments will be carried out using those strains which show the most promise. If resistant lines are obtained, studies will be carried out to determine the genetic basis of this resistance and to compare virus growth and distribution in resistant and susceptible individuals.

Honors and Awards:

None

Publications:

Gubler, D. J., and Bhattacharya, N. C.: Swarming and mating of Aedes (S.) albopictus in nature. Mosq. News, 32:219-223, 1972.

Gubler, D. J., Inui, T. S., Black, H. R., and Bhattacharya, N. C.: Comparisons of microfilaria density in blood sampled by finger-prick, venipuncture, and ingestion by mosquitoes. Am. J. Trop. Med. Hyg., 22:174-178, 1973.

Gubler, D. J., and Bhattacharya, N. C.: A survey for bancroftian filariasis in the Calcutta area. Ind. J. Med. Res., 61: 1973.

Serial No. NIAID 67 J

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

**Project Title:** Observations on a species of *Sarcocystis* with antigenic, morphologic and biologic similarities to *Toxoplasma*.

**Previous Serial Number:** None

**Principal Investigator:** Dr. Gordon D. Wallace

**Other Investigators:** None

**Man Years:**

| | NIAID Staff |
|---|---|
| Total: | 6/12 |
| Professional: | 6/12 |
| Other: | 0/12 |

**Project Description:**

Objectives

To elucidate the life cycle of a protozoan parasite presumed to be *Sarcocystis muris*, particularly to determine its relationship to *Toxoplasma gondii* and its potential to infect man.

Methods Employed

Sarcocysts morphologically similar to *Sarcocystis muris* were observed in mice after they were inoculated orally with *Toxoplasma*-like oocysts found in feces of a stray cat. Cats fed mice infected with the oocysts shed similar oocysts in their feces, thus establishing in the laboratory a life cycle similar to that of *Toxoplasma*. Serologic and histopathologic observations were made on mice and rats at various periods of time following inoculation with measured doses of oocysts. Observations were also made on cross-immunity between the parasite and *Toxoplasma* in experimentally infected cats and mice. To determine the prepatent and patent periods of infection with the parasite, which will be referred to as WC1170, feces of cats fed infected mice were collected daily and examined for the presence of the typical oocyst.

Major Findings

The oocyst of WC1170 appears to be morphologically identical to the oocyst of Toxoplasma. The prepatent and patent periods in 12 cats fed carcasses of infected mice average 5 and 13 days, respectively, compared to 4 and 12 days for Toxoplasma. Sera from cats with a patent WC1170 infection, with one exception, were negative at a 1:4 dilution in the Toxoplasma dye test. Two cats inoculated orally with large numbers of oocysts did not develop patent infections.

Experiments employing 13 cats demonstrated that infection with WC1170 conferred little or no immunity to Toxoplasma and vice versa, whereas a relatively solid immunity to WC1170 or Toxoplasma, respectively, was observed. It was also demonstrated that mice with chronic Toxoplasma infections could be infected with WC1170.

Sarcocysts were found in the musculature of about 40% of mice inoculated with 40,000 or more oocysts and examined 42 days or longer after exposure. However, only Toxoplasma-like cysts, presumed to be an early developing form of the sarcocyst, were found in mice examined less than 42 days after exposure. No cysts were found in mice inoculated with small doses (10-100) of oocysts, but such mice produced patent infection when fed to cats. Toxoplasma-like cysts in relatively small numbers were found in the brains of a small proportion of mice inoculated with large doses of WC1170 oocysts. Myositis was observed in most of the mice examined and was apparent before cysts were observed. Myocarditis was also observed but sarcocysts were never found in myocardium. Usually oocysts did not produce illness in mice unless relatively large doses were administered. Numerous attempts to infect susceptible mice with tissues from infected mice failed.

About 60% of mice inoculated with WC1170 oocysts developed low Toxoplasma dye-test titers (1:4-1:128). Indirect fluorescent antibody tests in which trophozoites harvested from mature WC1170 sarcocysts were used for antigen were done on sera from 14 mice infected with WC1170 oocysts. Results indicated that antibody to this stage of the parasite became detectable in mice about 30 days after exposure to oocysts and in some instances reached titers in the thousands. Such sera however reacted only in low dilution to Toxoplasma trophozoites in the same type of test. Sera with high Toxoplasma dye-test titers ($\geq$ 1:2000) from mice with chronic Toxoplasma infections did not react with the sarcocyst trophozoites.

To date, three laboratory rats have been exposed to WC1170 oocysts and examined at appropriate times for sarcocysts. However the only evidence of infection was the development of low dye-test titers. Muscle from one rat was fed to a susceptible cat but failed to produce a patent infection.

In summary, some stages of the WC1170 parasite are morphologically similar to those of Toxoplasma, the parasites share antigens, and have somewhat similar life cycles. However, WC1170 differs from Toxoplasma in the following ways: 1) lack of transmission between mice, at least within two months after infection; 2) very low virulence and only low Toxoplasma titers in mice; 3) Toxoplasma antibody usually not detectable in infected cats; 4) little or no immunity against Toxoplasma in cats following infection with WC1170.

Significance to Biomedical Research and the Program of the Institute

Information on this parasite is of interest because of its similarities to both Toxoplasma and Sarcocystis, and because the life cycle of Sarcocystis, which is known to infect man, has been obscure.

Proposed Course of the Project

There is a need for more information on the host range of the WC1170 parasite. Such information will be useful in elucidating its exogenous life cycle and evaluating its potential to infect man. Experimental infection in rats, guinea pigs, pigeons and Japanese quail are in progress or are being planned. Experimental infection of carnivores and primates will be considered. Systematic experiments on the endogenous life cycle in mice and cats, which will be of considerable biologic interest, are being planned in collaboration with Dr. Jack Frenkel of the University of Kansas, School of Medicine. There is also a need for a specific serologic test to detect infection with the parasite. This would be of considerable value in experimental work and also in detecting natural infection in man and lower animals. Attempts are being made to produce large numbers of trophozoites in mice for use in fluorescent antibody and dye tests.

Honors and Awards:

See NIAID 67D

Publications:

Wallace, G.D.: Sarcocystis in mice inoculated with Toxoplasma-like oocysts from cat feces. Science, 1973 (in press)

Serial No. NIAID 67 K

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The susceptibility of Pacific island rats to infection with Angiostrongylus costaricensis.

Previous Serial Number: None

Principal Investigator: Dr. Leon Rosen

Other Investigators: Dr. Gordon D. Wallace

Man Years:

| | NIAID Staff |
|---|---|
| Total: | 1/12 |
| Professional: | 1/12 |
| Other: | 0 |

Project description:

Objectives

To determine if A. costaricensis, a metastrongylid nematode of cotton rats in Central America, can produce patent infections in species of rats which occur on Pacific islands.

Methods Employed

Infective third stage larvae of A. costaricensis were obtained from Costa Rica and the life cycle established in cotton rats, Sigmodon hispidus, and aquatic snails, Biomphalaria glabrata, in the laboratory. Rattus rattus, R. norvegicus and R. exulans were then exposed to small doses (20-30) of A. costaricensis third stage larvae and observed for the excretion of first stage larvae. S. hispidus were exposed at the same time as controls. All of the rats were born and raised in the laboratory.

## Major Findings

Patent infections developed in three of eight R. rattus, one of three R. exulans that survived the prepatent period (23 to 27 days), and none of four R. norvegicus. Each of four S. hispidus that survived the prepatent period shed first stage larvae. The maximum number of larvae per gram of feces in S. hispidus ranged from about 5,000 to 30,000, whereas the range was about 100 to 200 in R. rattus and R. exulans. One R. exulans and one S. hispidus died before the 23rd day after exposure and two other S. hispidus died 33 and 57 days afterward. One R. rattus which did not excrete first stage larvae died 52 days after exposure. All of the surviving rats, with the exception of two R. rattus, were killed and examined for adult parasites two to four months after exposure. Rats that died from 17 to 57 days after exposure were examined as soon after death as possible. Up to 70% of worms administered to S. hispidus were recovered from the mesenteric arteries as adults, whereas a relatively small proportion of parasites were recovered from R. rattus and R. exulans. None were recovered from R. norvegicus.

## Significance to Biomedical Research and the Program of the Institute

Since A. costaricensis recently has been recognized as an important parasite of man it is of interest to learn if the rat species that occur in the Pacific area, where S. hispidus does not occur, could serve as definitive hosts. Another metastrongylid nematode of rats, A. cantonensis, is widespread in the Pacific area and it is conceivable that A. costaricensis could be introduced to this area or, in fact, already be present but not yet recognized.

## Proposed Course of the Project

Experiments are in progress in which rats of the species mentioned above were exposed to much larger numbers of A. costaricensis than in the first experiment. Survivors will be tested for immunity to A. cantonensis and eventually rats infected with A. cantonensis will be tested for susceptibility to A. costaricensis. The development of larvae of A. costaricensis and A. cantonensis in B. glabrata, and possibly in other molluscs, will be compared.

Honors and Awards: None

Publications:

Rosen, L.: Angiostrongyliasis. In Hubbert, W. T., McCulloch, W. F. and Schnurrenberger, P. R. (eds.): Diseases Transmitted from Animals to Man, Sixth Edition (in press)

LABORATORY OF IMMUNOLOGY

Summary ..... 1

Serial No.

140 - Receptors on Immunocompetent Cells ..... 6

141 - Lymphocyte Biology and the Interaction of Lymphocytes in the Immune Response ..... 15

142 - Genetic Control of Immune Response ..... 25

143 - Lymphocyte Interactions, Receptors and Functions ..... 32

144 - The Cellular Basis of Antibody Formation ..... 43

145 - Studies on Immunological Specificity ..... 53

146 - Chemical, Genetic, and Immunochemical Studies of Immunoglobulins: Use of Genetic Markers in Studies of Lymphoid Differentiation and the Regulation of Gene Expression and Immune Responses ..... 58

147 - Immunogenetics of Mouse Immunoglobulin ..... 67

148 - Genetic Control of Antibody Responses ..... 72

149 - Structural and Activity Studies on Immunologically Important Cells and Proteins ..... 76

PHS-NIH
Summary Statement
Office of the Chief
Laboratory of Immunology
July 1, 1972 through June 30, 1973

In the past year the Laboratory of Immunology has made considerable scientific progress and has maintained its high level of productivity. Dr. Ethan Shevach was appointed to the Laboratory as a Senior Staff Fellow and Drs. Donald Mosier and Michael Karpf joined the Laboratory as Research Associates. Dr. Michael Gelfand, an NIAID Special Fellow, and Dr. Salvatore Cara entered the Laboratory as Guest Workers and Drs. Shlomo Ben-Sasson and Ramesh Srivistava were appointed as Visiting Fellows. Dr. Steven Siegel who has been appointed to the early Commissioning Program, spent a three month elective period in the Laboratory.

One associate, Dr. John Stobo, and one fellow, Dr. Latham Claflin each completed their assignments at the beginning of this fiscal year. Both of these scientists had a substantial record of achievement during their tenure in the Laboratory.

The members of the Laboratory continue to play an important role in the immunologic community. Dr. Paul serves on the editorial boards of the Journal of Immunology, Immunochemistry, the Scandinavian Journal of Immunology and Immunological Communications. He is also a member of the board of reviewers of Federation Proceedings as are Drs. Green and Inman, while Dr. Mage is a member of the editorial board of that journal and of Immunochemistry. Dr. Merchant is a member of the editorial board of Infection and Immunity.

Dr. Mosier served as a session chairman at the annual meeting of the American Association of Immunologists and Dr. Paul chaired a session at a meeting on Microenvironmental Aspects of Lymphoid Tissue in Dubrovnik, Yugoslavia. Dr. Paul participated at a European Molecular Biology Organization Workshop in Immunology in Oxford, Dr. Mage attended a meeting at the Pasteur Institute in honor of the 150th anniversary of the birth of Louis Pasteur, and Dr. Green has been invited to participate in an international workshop on Cell Mediated Immunity at Vence, France. In addition, Dr. Paul presented major talks at the 26th Annual Basic Science Symposium of the M.D. Anderson Hospital and Tumor Institute in Houston and at a workshop on New Approaches for Inducing Natural Immunity to Pyogenic Organisms in Winter Park, Florida. Dr. Shevach was a major speaker at the Gordon Conference on Immunology and Immunochemistry. Members of the Laboratory have given seminars at many major Universities in the United States and Europe.

Dr. Mage is a member of the Allergy and Immunology Study Section and Dr. Merchant was a member of the Secretariat and Dr. Paul of the Directing Group of the NIAID Task Force on Immunology and Disease. Finally, Dr. Merchant continues to serve as the Equal Employment Opportunity Counselor for NIAID.

The Laboratory of Immunology has as its basic goal the exploration of fundamental mechanisms in cellular immunology, immunogenetics, immunochemistry, immunopathology and tumor immunology. To these ends, studies of the ontogeny and heterogeneity of lymphocytes, of the nature and function of antigen-binding membrane receptors of lymphocytes, and of the mechanisms of tolerance induction and lymphocyte interactions are being actively pursued. Major efforts are being exerted to elucidate the nature, genetics and mode of function of specific immune response gene products. Mechanisms of cell mediated cytotoxicity, of the nature of the genetic and chemical control of immunoglobulin synthesis and variability and of the activation of immunocompetent cells are being intensely studied.

Ontogeny of Immunoglobulin Bearing Lymphocytes

The development of bone marrow derived (B) lymphocytes has been difficult to study in mammals because no single central lymphoid source organ has yet been identified. The understanding of the development of these cells is of considerable importance as they are the precursors of antibody secreting cells. Consequently, studies of the appearance of lymphocytes with easily detectable surface immunoglobulin (Ig) during the perinatal period in mice were performed. These cells, which are B lymphocytes, are found in very small numbers at the time of birth but their frequency increases markedly during the first several days of life. Indeed, the increase in the number of Ig bearing cells during the first day of life is approximately 6 fold. As no organ supplying Ig bearing cells to the neonatal spleen has been identified, the possibility was strong that some B cell differentiation occurred within the spleen. Consequently, cell suspensions from neonatal spleen and liver were cultured, in vitro, for 24 hours and the number of Ig bearing cells then determined. A marked increase (2 to 4 fold) in the frequency of such cells was noted and this increase could be inhibited by mitomycin C indicating a requirement for cell division. Pretreatment of neonatal spleen cells with anti-Ig serum and complement diminishes the number of Ig bearing cells after 24 hour culture suggesting that many of the precursors of these cells already have some surface Ig. This system provides a valuable tool to further study the physiology of B lymphocyte differentiation (Gelfand, Paul, Asofsky (LMI/NIAID) and Lawton (Univ. of Alabama)).

Ontogeny of T Lymphocytes

T Lymphocytes develop within the thymus from more primitive precursors derived from hematopoietic tissue. This differentiation process is associated with acquisition of immunocompetence on the part of the thymocytes and T lymphocyte. Although these events are very poorly understood, they are crucial to the understanding of the developmental aspects of immunocompetence. In an effort to delineate these events, the capacity of fetal thymocytes to respond to allogeneic and syngeneic lymphoid cells and to various T lymphocyte mitogens was evaluated. The striking finding was made that a peak in responsiveness in several of these systems, particularly in allogeneic mixed lymphocyte reactivity and response to phytohemagglutin, was observed at 18 to 19 days of gestation. These responses and the syngeneic mixed lymphocyte reactivity diminish in magnitude subsequently although allogeneic mixed lymphocyte

responsiveness and reactivity to other T lymphocyte stimulants reappear in the second week after birth. This result suggests that in late fetal life an immunologic mechanism capable of suppressing some immunocompetent thymocytes appears. One possible function of such a phenomenon would be to eliminate cells responsive to host components (Mosier).

## SPECIFIC INDUCTION OF TOLERANCE IN B LYMPHOCYTE POPULATIONS

The 2,4-dinitrophenyl (DNP) derivative of the copolymer of D-glutamic and D-lysine (D-GL) is a potent tolerogen. Mice and guinea pigs which have been pretreated with DNP-D-GL make little or no anti-DNP antibody response upon subsequent challenge with DNP on unrelated carriers such as hemocyanin. The hapten specificity of most immune responses is largely due to the B lymphocytes, which are the precursors of the antibody secreting cells. Thus, the hapten (DNP), specificity of this tolerance suggests that it is primarily a function of B lymphocytes. In order to demonstrate this, normal mice, mice which had been thymectomized, irradiated and reconstituted with bone marrow only (TxBM) and TxBM mice which also received thymocytes were treated with tolerogenic doses of DNP-D-GL and the capacity of their spleen cells to respond to DNP-hemocyanin in lethally irradiated hosts measured. The recipients also received normal thymocytes so that the full potentiability of the transferred B lymphocytes could be evaluated. It was found that spleens from tolerized normal, TxBM and TxBM mice reconstituted with thymocytes, all were tolerant in recipients and expressed the same degree of tolerance demonstrating that tolerance to DNP-D-GL is largely in the B lymphocyte pool and that thymus-derived lymphocytes play little if any role in the induction of the tolerant state. These experiments provide evidence that certain molecules may lead to direct inactivation of B lymphocytes and will be of value in designing approaches to render B lymphocytes tolerant while maintaining normal T lymphocyte responses (Breitner, Cohen and Paul).

## SPECIFICITY OF ALLOANTISERA WHICH BLOCK IMMUNE RESPONSE GENE FUNCTION

In guinea pigs, immune responses to certain simple antigens are under the control of single autosomal dominant immune response (Ir) genes which are linked to the genes controlling histocompatibility antigens. Thus, strain 2 guinea pigs respond to the dinitrophenyl (DNP) conjugate of the copolymer of L-glutamic acid and L lysine (GL) and to the copolymer of L-glutamic acid and L-alanine (GA), while strain 13 guinea pigs are unresponsive to both antigens. On the other hand, strain 13 guinea pigs respond to the copolymer of L-glutamic acid and L-tyrosine (GT), to which strain 2 guinea pigs fail to respond. $(2\times13)F_1$ guinea pigs are responsive to each of these antigens. The capacity of immune $F_1$ lymphocytes to respond to either DNP-GL or to GA is blocked by anti-2 alloantisera, but not by anti-13 sera while responses to GT are blocked by anti-13 sera but not anti-2 sera. These sera thus prevent Ir gene function. Whether the sera do so by directly binding to Ir gene products on the cell surface or by binding to histocompatibility antigens and blocking Ir gene products sterically is uncertain. In order, to examine this crucial question, the response of lymphocytes from outbred GA responsive guinea pigs was tested. Although many outbred guinea pigs are $2^+$, $GA^+$, $DNP\text{-}GL^+$, as are strain 2 guinea pigs, some are $2^-$, $GA^+$, $DNP\text{-}GL^-$. If alloantisera block the re-

sponse to GA because they contain antibodies specific for the GA Ir gene product, anti-2 alloantisera should block the GA response of lymphocytes from both $2^+$ and $2^-$ animals. However, only the response of lymphocytes from $2^+$ animals was blocked. These, and other experiments, suggest that alloantisera block Ir gene function by interacting with histocompatibility antigens. This, in turn, implies a stable linkage on the cell surface between histocompatibility antigens and Ir gene products. These experiments are of great importance in the understanding of the antigen-recognition and activation mechanisms of T lymphocytes (Shevach, Paul and Green).

ISOLATION OF A PEPTIDE BEARING a LOCUS ALLOTYPE (V REGION) ANTIGENIC DETERMINANTS

The capacity of antibody molecules to bind to the antigens for which they are specific is dependent on the structure of the variable (V) regions of heavy (H) and light (L) polypeptide chains. The $V_H$ genes (the genes controlling the V regions of H chains) of rabbits appear to exist in allelic forms as evidence by polymorphism at the a locus. In order to elucidate the structural basis of this polymorphism and to further substantiate that this simple Mendelian genetic marker is indeed in the $V_H$ region, H chains from anti-polysaccharide antibodies of restricted heterogeneity produced by rabbits homozygous for the a3 marker were digested with trypsin. Peptides capable of binding to anti-a3 antibody were isolated and purified. A peptide of 25 to 30 residues has been obtained which reacts specifically with anti-a3 sera. Studies of the sequence of this peptide and its precise location in the H chain are now in progress. These studies will allow a determination of the genetic and chemical basis of the a locus allotypy. This is of crucial importance to the understanding of the genetic basis of antibody diversity as the existence of a genetic marker, obeying Mendelian laws and controlling the structure of the $V_H$ region, imposes major limitations on the number of $V_H$ genes in the genome (M. Carta, R. Mage and E. Appella, (LCB/NCI)).

LINKAGE OF A GENE CONTROLLING AN IDIOTYPIC SPECIFICITY (VARIABLE REGION MARKER) TO THE GENE COMPLEX CONTROLLING IMMUNOGLOBULIN ALLOTYPIC SPECIFICITIES (CONSTANT REGION MARKERS) IN MICE

Several myeloma proteins of BALB/c mice bind phosphoryl choline and, of these, several share a common idiotypic determinant and have marked structural similarity. The idiotypic determinant, denoted as the T-15 idiotype, is present on the variable domain of immunoglobulin and thus is a variable region genetic marker. The existence of this idiotypic specificity has now been demonstrated in both normal and immune serum of some, but not all, strains of mice. Thus, the serum of BALB/c mice contain the T-15 idiotype while C57BL/6 sera lack this idiotype. The analysis of a series of mouse strains derived from BALB/c x C57BL/6 matings reveals that the inheritance of the T-15 idiotype is linked to the inheritance of immunoglobulin heavy (H) chain allotypic determinants from the BALB/c. This demonstrates linkage between genes controlling H chain constant and variable regions and provides a powerful system for the analysis of genetic control of immunoglobulin variable regions (Lieberman and Potter, (LCB/NCI).

IDENTIFICATION OF MULTIPLE LECTINS ASSOCIATED WITH POKEWEED (PHYTOLACCA AMERICANA)

A mitogen isolated from pokeweed has been described which has the unusual property of activating both bone marrow-derived (B) and thymus-derived (T) lymphocytes. As other mitogens are restricted in their activating potential to either T or B lymphocytes, this mitogen has unique interest. It has now been shown that a family of 5 distinct lectins (Pa 1-5) exist in extracts of pokeweed. These lectins have been purified and characterized. One of these lectins (Pa-2) corresponds to the previously described pokeweed mitogen and this lectin, as well as Pa-1 and Pa-3, is highly stimulatory for mouse spleen cells. Pa-4 and Pa-5 are also mitogenic but much higher concentrations are required. Under physiologic conditions the mitogenic pokeweed lectins each consist of a single polypeptide chain, suggesting that they may have only one binding site. As most other cell stimulants are multivalent, an analysis of the mechanism by which Pa-2 stimulates lymphocytes promises to be particularly fruitful (Waxdal).

IDENTIFICATION OF TYPE OF MONONUCLEAR CELLS IN HUMAN LESIONS

A number of receptors have been discovered on the surface of monocytes and lymphocytes which allow the characterization of these cells. Monocytes have a receptor for the third component of complement (C) and for immunoglobulin G (IgG). B lymphocytes also have a receptor for C3 and a receptor for aggregated $\gamma$ globulin. T lymphocytes lack both of these receptors. However human T cells bind sheep erythrocytes. Techniques are now available to detect such receptors on mononuclear cells both in suspension and in frozen sections. Red blood cells coated with either IgG antibody or C3 are the reagents used for these studies. These methods have now been successfully applied in the study of human pathological material. Thus, the mononuclear infiltrates in the salivary glands of patients with Sjogren's Syndrome are predominantly B cells. The mononuclear cell infiltrate in a number of dermatological diseases have also been investigated. Finally, these techniques have been applied to the study of human lymphomas. Sections from two patients with nodular lymphoma show that the malignant cells is a B cell. In two patients with leukemic reticuloendotheliosis, the malignant cells had the IgG receptor characteristic of monocytes. Also in a patient with histocytic medullary reticulosis, the abnormal cell possessed the IgG receptor characteristic of monocytes. The use of these techniques should lead to a better understanding of the role of mononuclear cells in immunopathologic lesions. These studies should also allow a new classification of human lymphomas which may have far reaching prognostic and theraputic implications (Green, Shevach, Edelson (NCI), Chused (NIDR) and Jaffe (NCI)).

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Receptors on Immunocompetent Cells

Previous Serial No.: Same

Principal Investigator: Dr. William E. Paul

Other Investigators:

| | | |
|---|---|---|
| Project Subtitle | I: | Dr. B. E. Cohen, LI/NIAID<br>Dr. A. S. Rosenthal, LCI, NIAID |
| Project Subtitle | II: | Dr. S. Z. Ben-Sasson, LI, NIAID<br>Dr. B. E. Cohen LI, NIAID<br>Dr. C. A. Janeway, Jr.,LI,NIAID<br>Dr. M. Karpf, LI,NIAID |
| Project Subtitle | III: | Dr. J.C.S. Breitner, LI, NIAID<br>Dr. B. E. Cohen, LI, NIAID<br>Dr. J. E. Davie, Dept. of Path. Washington Univ. School of Medicine, St. Louis, Missouri |

Cooperating Units: Department of Pathology, Washington University School of Medicine, St. Louis, Missouri
Laboratory of Clinical Investigation, NIAID

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 5 7/12 | | | 5 7/12 |
| Professional | 4 1/12 | | | 4 1/12 |
| Other | 1 6/12 | | | 1 6/12 |

Project Subtitle I: Antigen-binding By Macrophages

Project Description:

Objectives:

The initial step in many immune responses involves interaction of antigen with macrophages which act as "presenting" cells in that antigen associated with these cells is especially effective in the activation of specific lymphocytes. Although the macrophage is known to be capable of binding various antigens to its surface, the molecular mechanism of this binding is poorly understood. In these studies, the relative roles of cytophilic antibody, "non-specific" membrane sites and immune response (Ir) gene products in antigen-binding were evaluated.

Methods Employed:

Macrophage rich populations obtained from oil induced peritoneal exudates were incubated with radioactive or non-radioactive antigens under a variety of conditions. After extensive washing, the total amount of antigen associated with the cells was determined by measuring radioactivity and the capacity of this antigen to induce an immune response was evaluated by incubating the macrophages, after treatment with mitomycin C, with populations of immune lymphocytes. The response of the latter cells was determined by their degree of incorporation of tritiated thymidine ($^{3}$H-TdR) into DNA 72 hours after initiation of cultures.

Major Findings:

Macrophages from guinea pigs immunized to 2,4-dinitrophenyl (DNP) guinea pig albumin (GPA), bind antigen efficiently at both low and high antigen concentrations. When incubated with 0.01 µg of DNP-GPA per ml, the binding is inhibitable by 90% or more by DNP on unrelated carriers, such as ovalbumin (DNP-OVA), or by anti-immunoglobulin sera. In addition, the capacity of these macrophages to stimulate the response of specific lymphocytes is also inhibited if either DNP-OVA or anti-immunoglobulin is present during incubation with low concentrations of DNP-GPA. This suggests that cytophilic antibody on the macrophage surface is the predominant macrophage "receptor" when low antigen concentrations are used. This was verified by the demonstration that macrophage preparations from non-immune guinea pigs bound little DNP-GPA when it was present at $10^{-2}$ µg/ml. These cells caused little or no stimulation of immune macrophages under such conditions. What little binding was achieved by non-immune macrophages at low DNP-GPA concentrations could not be blocked by DNP-OVA or anti-immunoglobulin. The binding and stimulatory capacity of non-immune macrophages which are confronted with low antigen concentrations can be markedly enhanced if these cells are preincubated in anti-DNP serum.

When macrophages are incubated with high concentrations of DNP-GPA ($10^{+2}$ µg/ml), both non-immune and immune macrophages are equally efficient at binding antigen and at subsequently stimulating immune lymphocytes. Their binding and stimulatory activity is blocked little or not at all by excess DNP-OVA or anti-immunoglobulin serum and incubation of non-immune macrophages in immune serum does not increase their activity. This result indicates that cytophilic antibody makes little contribution to antigen-binding by macrophages at high antigen concentrations. Under these conditions, so-called "non-specific membrane sites" are the predominant antigen binding receptors on the macrophage surface.

An attempt to evaluate the function of immune response gene products in macrophage interaction with antigen was made. The responses to the DNP conjugate of the copolymer of L-glutamic acid and L-lysine (DNP-GL), and to low doses of bovine serum albumin (BSA) are controlled by immune response genes linked to strain 2 histocompatibility genes. Thus, strain 2 and (2x13)$F_1$

guinea pigs respond to DNP-GL and low doses of BSA while strain 13 guinea pigs fail to respond. Moreover, an in vitro response of immune $(2x13)F_1$ lymphoid cells to these antigens is blocked by anti-2 alloantisera but not by anti-13 serum. Similarly, responsiveness to the copolymer of L-glutamic acid and L-tyrosine (GT) and to DNP-GPA is linked to strain 13 H antigens. Strain 13 and $(2x13)F_1$ guinea pigs respond to these antigens and anti-13 alloantiserum blocks the response of $(2x13)F_1$ immune lymphoid cells. In order to determine the role of Ir gene products in antigen-binding to macrophages, peritoneal exudate cells from non-immune strain 2 and strain 13 guinea pigs were compared in their capacity to bind $^{3}$H-DNP-GL, $^{125}$I-BSA, $^{125}$I-GT and $^{125}$I-DNP-GPA at various ligand concentrations. No difference in antigen-binding for any of these antigens was observed. In addition, the capacity of $^{3}$H-DNP-GL to bind to non-immune $(2x13)F_1$ macrophages in the presence of anti-2 and anti-13 serum was studied. No difference was noted. These results strongly suggest that Ir gene products have little or no role in antigen-binding to macrophage surfaces.

Significance to Biomedical Research and to the Program of the Institute:

The macrophage appears to be a crucial cell in determining the capacity of the immune apparatus to recognize and respond to foreign antigens of both experimental and pathogenetic significance. These studies provide important data on the nature of this interaction and should be of value in designing strategies for optimal immunization in clinical and public health settings or alternatively, for obtaining tolerance.

Proposed Course of Project:

This project will be pursued in two general ways. Attempts to determine the chemical nature of the "non-specific" membrane receptor and its relation to histocompatibility antigens will be undertaken. In addition, the morphologic characterization of this membrane binding will be pursued.

Project Subtitle II: Antigen-Binding Receptors of Thymus-Derived (T) Lymphocytes

Project Description:

Objectives:

T lymphocytes play an important role in both cellular immune and antibody responses. They bear specific receptors which are responsible for their immunologic specificity but the chemical nature of these receptors is unknown. Thus far, direct attempts to characterize these receptors as being immunoglobulin in nature have led to equivocal results as have attempts to show direct antigen binding. Although continued efforts to directly analyze antigen-binding receptors are still in progress, indirect studies based upon the blocking of T lymphocyte activation in vitro are also being pursued.

Studies of in vitro responses of guinea pig T lymphocytes have concentrated on the specificity of elicitation of these responses. In conventional immunization with hapten-protein conjugates, the in vitro response is highly carrier or "conjugate" specific. Thus, the response of T lymphocyte preparations from guinea pigs immunized to DNP-GPA can be elicited by DNP-GPA but DNP on other proteins causes little or no response. On the other hand, it has been observed that the univalent ligand ε-DNP-L-lysine and DNP on the unrelated protein ovalbumin (OVA) block the activation of T lymphocytes. This latter observation suggested that the receptors might have considerable specificity for the DNP group. It has now been shown that the capacity of DNP-OVA to inhibit T lymphocyte responses is due to blocking of binding of DNP-GPA to "immune" macrophages, and not by inhibiting binding to T lymphocytes. Thus, incubation of "immune" macrophages with DNP-OVA prior to the addition of low concentrations of DNP-GPA blocks the capacity of the macrophages to activate immune lymphocytes. On the other hand, incubation of lymphocytes in excess DNP-OVA prior to the addition of macrophages which have already bound DNP-GPA has no inhibitory effect. Thus, in such a system there are few, if any, hapten-specific T lymphocytes.

Recently, a novel immunization protocol has been introduced which may activate hapten-specific T lymphocytes. Killed mycobacteria are directly conjugated with 1-fluoro-2,4-dinitrobenzene to yield DNP-mycobacteria. These organisms are emulsified in incomplete Freund's adjuvant. Lymphocytes from guinea pigs immunized with DNP-mycobacteria are stimulated in vitro by DNP-GPA, DNP-OVA, DNP-hemocyanin and DNP-bovine gamma globulin but not by DNP on non-immunogenic carriers. This system may allow a detailed study of structural requirements for T cell activation and a precise evaluation of the relation of T cell receptors and Ir gene products.

Finally, direct attempts to isolate antigen-binding receptors of T lymphocytes have been initiated. The approach has been to prepare purified populations of T lymphocytes from which B lymphocytes and macrophages are excluded. Cells from immune and non-immune animals are labelled with $^{125}I$ by the lactoperoxidase procedure and surface proteins are extracted with non-ionic detergents or, alternatively, are allowed to "shed" into culture media. The capacity of these proteins to bind antigen was determined by mixing with antigen and analyzing by sucrose density gradient ultracentrifugation or by the capacity of $^{125}I$ material to bind to agarose bead columns to which antigen had been linked. Preliminary results reveal small amounts of specific binding but indicate that enrichment of antigen-specific T cells would be highly desirable so that the ratio of specific to non-specific material could be increased.

Significance to Biomedical Research and the Program of the Institute:

The nature of the antigen-binding receptor of the T lymphocyte is among the most controversial and important problems in contemporary immunology. In attempts to specifically increase or diminish the degree of T lymphocyte activation in clinical settings, knowledge of the chemical nature of the receptor and of the structural and specificity requirements for its activation will be of great value.

Methods Employed:

In vitro activation of guinea pig and mouse lymphocytes has been measured using the ability of antigen-stimulated lymphocytes to incorporate $^{3}$H TdR into DNA. The ability of various agents such as haptens, anti-immunoglobulin sera and anti-histocompatibility sera to block such activation and, similarly, the relative capacity of various antigens to stimulate activation have been studied in an attempt to indirectly characterize the receptor.

In addition, iodination of surface membrane proteins by the lactoperoxidase procedure has been employed in attempts to isolate material from T cells with antigen-binding activity.

Major Findings:

A system for evaluating in vitro response of mouse spleen cell cultures was established. This system involves the use of 5 x $10^{5}$ cells in a 0.2 ml volume microculture system in the absence of any serum source. The medium used generally includes 2-mercaptoethanol. Under these conditions both mitogen and antigen responses can be obtained. The responses are measured by the incorporation of $^{3}$H-TdR after 72 hours. Spleen cells from mice immunized with DNP-hemocyanin respond quite well. These responses can be obtained when the vast majority of B lymphocytes have been removed from the cell population by passage over columns bearing antigen-antibody complexes. B lymphocytes are bound by such columns because they bear a receptor for aggregated immunoglobulin. T lymphocytes which lack such a receptor, pass through the column. Furthermore, the number of macrophages can be markedly diminished in such populations by the use of adherence procedures. In such a situation, the in vitro response primarily represents responses of specific T lymphocytes. In preliminary studies, purified antibodies directed at κ light chains markedly diminish the response to antigen without impairing responses to concanavalin A or phytohemagglutinin. If these results can be confirmed and extended, they will support the contention that immunoglobulin has an important role in antigen recognition by T lymphocytes.

In addition, this system has been employed to evaluate immune response gene function in the mouse. C57BL/10 mice (B10 mice) respond to the branch chain polymer of L-tyrosine, L-glutamic acid, D-L-alanine and L-lysine ((T,G)-A--L) while the congenic strain B10·A fails to respond. The Ir gene controlling this response is linked to H-2 genes. The H-$2^{b}$ type is associated with responsiveness while the H-$2^{a}$ type is associated with unresponsiveness. Spleen cells from B10 mice immunized to (T,G)-A--L are stimulated to synthesize DNA in vitro by (T,G)-A--L and this response is blocked by an anti B10 alloantisera prepared in B10·A mice but not by an anti B10·A serum prepared in B10 mice. Because of the availability of congenic recombinant inbred mouse strains, this system is a particularly powerful one to analyze T cell receptors and Ir gene products.

Proposed Course of Project:

Efforts to extend each of the current avenues will be continued and new approaches to the direct demonstration of antigen-binding by T lymphocytes will be employed. A particular goal will be to delineate the relationship between T lymphocyte receptors and Ir gene products.

Project Subtitle III: Antigen-Binding Receptors of Bone Marrow Derived (B) Lymphocytes

Project Description:

Objectives:

B lymphocytes have been demonstrated to bear surface immunoglobulin and this immunoglobulin functions as the antigen recognition mechanism of these cells. In the current studies, further characterization of the number and type of receptor bearing B lymphocytes in immune and tolerant states was undertaken. In particular, a model of B lymphocyte tolerance in mice was evaluated for the presence or absence of receptor bearing cells and the capacity of thymus-dependent antigens to expand the population of receptor bearing B lymphocytes in thymus-deprived animals was studied.

Methods Employed:

The enumeration of antigen binding B lymphocytes involved the incubation of lymphocyte populations with $^{125}$I-DNP-GPA of high specific activity. After 30 minutes at 4°, the cells were extensively washed and radioautographs prepared. The number of antigen-binding cells was determined by direct counting.

Major Findings:

The DNP conjugate of the copolymer of D-glutamic acid and D-lysine has previously been shown to be a potent tolerogen in the guinea pig. The guinea pig studies suggested that tolerance was primarily induced in the B cell pool because it was DNP specific. Nonetheless, the demonstration of the B cell nature of this tolerance required study in an animal in which appropriate deprivation and reconstitution studies could be carried out. It was first shown that DNP-D-GL in doses of 100 µg or greater was a potent tolerogen in mice and that animals which had received 1 to 3 mg of DNP-D-GL one week prior to immunization with DNP hemocyanin made virtually no detectable anti-DNP antibody in response to that immunization. It was then shown that spleen cells from tolerant AKR mice were less efficient than control spleens in reconstituting lethally irradiated syngeneic mice for a response to DNP-hemocyanin. It was of interest that although the tolerant state in the donor was profound in degree, the tolerance in the recipient was less striking in quantitative terms.

The induction of tolerance in the B lymphocyte pool of thymus deprived mice was demonstrated as follows. AKR mice were thymectomized as adults, lethally irradiated and reconstituted with bone marrow cells. These mice (referred to as TxBM mice) were treated with DNP-D-GL and their spleen cells transferred to irradiated recipients together with thymocytes. The recipients were immunized with DNP-KLH and their responses compared to mice which had received spleen cells from non-tolerized TxBM donors. In 4 experiments, a degree of tolerance similar to that observed by the transfer of intact tolerized spleen cells was observed. In addition, reconstitution of the TxBM mice with syngeneic thymocytes prior to tolerance induction did not increase the degree of transferable tolerance. These results establish that DNP-D-GL can induce a tolerant state in the B cell pool without the participation of T cells.

Spleen cells from control and tolerized normal and TxBM mice were examined for DNP specific antigen-binding cells one week after tolerance induction. Tolerant intact animals had 6 to 10 fold fewer DNP binding cells than control animals. Similarly tolerized TxBM animals had many fewer antigen-binding cells than non-tolerant TxBM mice.

A second issue involving changes in antigen-binding B lymphocytes was whether the B lymphocytes of thymus deprived mice made any response to thymus dependent antigens. TxBM AKR mice were immunized with DNP-hemocyanin or DNP-BSA emulsified in complete Freund's adjuvant. The number of antigen-binding and antibody secreting cells was determined before and after secondary challenge. It was observed that in non-immune control and TxBM AKR mice, most antigen-binding cells had surface IgM. After immunization of control animals, a marked expansion in the number of antigen-binding cells was noted and a substantial fraction of these cells had antigen-binding receptors of the IgG class. On secondary challenge, these animals produced significant amounts of IgG anti-DNP antibody. Surprisingly, TxBM mice displayed a similar expansion in the number of antigen-binding cells after primary and secondary challenge. However, in these mice, the great majority of such cells had surface IgM. All the antibody producing cells noted in such animals secreted anti-DNP antibody of the IgM class. This result indicates that the role of the T lymphocyte in B cell activation may be to control differentiation, particularly the "switch" from IgM to IgG, to a much greater extent than proliferation.

Significance to Biomedical Research and the Program of the Institute:

These studies of tolerance induction and of activation of B lymphocytes and of the changes in frequencies of cells bearing specific receptors bring us closer to an understanding of the regulation of B lymphocyte function. This is important in all antibody forming responses and is particularly germane when we deal with thymus independent antigens, such as bacterial polysaccharide in which T lymphocytes make little contribution to the response.

Proposed Course of Project:

Future studies of the status of B lymphocytes in tolerant animals will concentrate in determining whether tolerogen remains on the surface of antigen-binding cells, whether these cells are "modulated" or whether they are irreversibly inactivated or dead. In the further exploration of the expansion of specific B cell populations in the absence of T cells, an analysis of the functional potentialities of IgM bearing B cells is planned. In particular, the question of whether these cells can generate IgG producing cells if T lymphocytes are subsequently provided will be studied.

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Serial No. NIAID - 141
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Lymphocyte Biology and the Interaction of Lymphocytes in the Immune Response

Previous Serial No.: Same

Principal Investigator: Dr. William E. Paul

Other Investigators:

| | |
|---|---|
| Project Subtitle I: | Dr. C.A. Janeway, Jr. |
| Project Subtitle II: | Dr. D.E. Mosier |
| | Dr. H.C. Morse, Jr., LMI/NIAID |
| | Dr. B.E. Cohen |
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| | Dr. A. Steinberg, NIAMDD |
| Project Subtitle III: | Dr. M. Gelfand |
| | Dr. G. Elfenbein |
| | Dr. J.A. vanBoxel |
| | Dr. R. Asofsky, LMI/NIAID |
| | Dr. A. Lawton, Univ. of Alabama |
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| | Dr. P.R.B. McMaster, Jr., LMI/NIAID |
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Cooperating Units: LMI/NIAID
Arthritis Branch, NIAMDD
Dept. of Pediatrics, Univ. of Alabama School of Medicine, Birmingham, Alabama
Dept. of Pathology, Washington Univ. School of Medicine, St. Louis, Missouri

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 6-5/12 | | 1 | 7-5/12 |
| Professional | 3-5/12 | | 1 | 4-5/12 |
| Other | 3 | | | 3 |

Project Subtitle I: Specificity and Radiation Sensitivity of T and B Lymphocytes

Project Description:

Objectives:

Immune responses to most antigens involve the cooperation of two major classes of lymphocytes, the thymus derived (T) and bone marrow derived (B) lymphocytes. While the major function of B cells is antibody synthesis and production, T cells not only are essential in "helping" the B cell to become activated by antigen, but are also the principal cells involved in antigen recognition and all types of cell mediated immunity. The objective of this work has been to study T cells for their helper function, in order to achieve three goals: 1) An understanding of the functional specificity of T cells. 2) What types of antigens do or do not activate T cells. 3) How T and B cells cooperate to produce antibody.

Methods Employed:

Irradiated mice are reconstituted with spleen cells from normal or immunized syngeneic donor mice. The recipient mice are subsequently boosted with antigen. The amount of antibody produced is a function of the activity of the cooperating cell types.

Major Findings:

One of the major distinctions between T and B cells, which has been of great practical value in studying their properties separately, lies in their functional specificity. Thus, upon immunization with hapten-protein carrier conjugates, B cells specific for both the hapten and for the protein carrier are readily detectable both as antigen binding cells and by their ability to produce antibody of the appropriate specificity. T cells on the other hand are extremely difficult to detect as antigen binding cells; therefore, one detects them by their helper function. While carrier specific helper cells are readily demonstrable, hapten-specific help has been extremely difficult to detect. This distinction is of some importance for several reasons. First, it is of practical use in studying T and B cell function. Second, if hapten specific T cells can be described, then the precise specificity of their receptors can be determined using chemically defined hapten inhibitors. If that specificity is identical to the specificity of serum antibody, then it would be very likely that the T cell receptor is immunoglobulin. On the other hand, if the specificity is different from serum Ab, then the T cell receptor is either an unusual class of Ig or an altogether different molecule. Finally, as one is dealing with a functional response, the cell must not only recognize, but also be triggered by the antigen in question. In last year's report, one hapten-specific helper effect was shown in cell transfer experiments to be mediated by hapten-specific serum antibody. In this report, a second reported helper effect is also shown to be mediated by antibody. DNP-BSA primed mice boosted with DNP-OVA make more

anti-OVA than mice primed with BSA alone. Using a dual transfer technique, in which OVA primed B cells are transferred along with anti-DNP antibody or DNP primed cells, it was shown that this hapten-specific helper effect was also due to anti-DNP antibody. No evidence for DNP-specific helper cells was found. In recent experiments (Serial 140, Subtitle II), guinea pigs were immunized with DNP conjugated to Mycobacterium tuberculosis. Cells from these animals have been shown to transform with hapten on any immunogenic carrier, but not with the carrier alone. Studies are now in progress to determine if these cells are DNP-specific and if they can function as helper cells in both mouse and guinea pig systems.

Protein-specific helper cells have been studied in a variety of systems. It has been shown that, early after boosting, doubling the number of helper cells leads too close to a four-fold increase in antibody produced. The reason for this is not yet clear. In attempting to alter this phenomenon, it has been shown that helper cells are very much more sensitive to the action of anti-lymphocyte serum in vivo, than are B cells. B cells are very sensitive to x-ray in vitro, 50 Rads inactivating half of B cells. 200 Rads in vitro inactivates half of helper cells. It has not been possible to confirm some previous reports of radioresistance of helper cells. However, if antigen is present at the time of irradiation, radioresistant helper effects can be obtained.

Significance to Biomedical Research and the Program of the Institute:

T cells are the major lymphocyte class involved in antigen recognition and all phases of cell-mediated immunity. A precise knowledge of T cell receptor specificity is of extreme importance in understanding immunological recognition. The interactions of T cells with B cells and possibly with other T cells are very important in all phases of immunity.

Proposed Course of Project:

Hapten-specific T cells will be studied for their precise specificity, the effect of antigen configuration on T cell activation, the effect of genetic factors on responses of such cells, and whether such cells show helper effects. Further efforts are underway to determine the cause of the apparent positive interaction between helper cells.

Project Subtitle II: Functional Heterogeneity of Thymus Cell Subpopulations

Project Description:

Objectives:

To examine the ability of mouse thymic lymphocytes to respond to specific antigenic stimulation or nonspecific phytomitogen stimulation at different times in ontogeny and following inductive interaction with thymic epithelium. These studies are designed to further understanding of: 1) the differentiation of thymus cells into functionally distinct subpopulations, 2) the maturation of "T" lymphocyte function as observed in peripheral lymphoid organs, and

3) the nature of the interaction between lymphoid stem cells and thymic epithelium.

Methods Employed:

Thymuses were removed from BALB/c inbred mice ranging in age from 14 days of gestation to adulthood and cultured either with mitomycin-treated allogeneic or syngeneic spleen cells to give a mixed lymphocyte reaction (MLR), or with phytohemagglutinin (PHA), concanavalin A (Con A), or pokeweed mitogen (PWM). The ability of thymocytes of varying ages to respond to those stimuli was quantitatively determined by measuring the rate of DNP synthesis. Thymocytes from (NZB x NZW)$F_1$ mice were assayed in the same way.

Thymocytes were also cultured in the presence of an established cell line derived from thymic epithelium, and changes in their functional activity measured by the above methods as well as by migration patterns of $^{51}Cr$-labelled cells and the ability to induce graft-vs-host reactions in $F_1$ neonatal recipients.

The properties of the thymic epithelial cell line were further studied by inoculating cells grown in culture into syngeneic BALB/c mice and adapting the cells to grow as a solid, transplantable tumor. The ability of the thymic epithelial tumor to reconstitute the immune response of athymic nude mice or neonatally-thymectomized mice was evaluated using graft rejection, humoral antibody formation, and a variety of correlates of cellular immunity to assess reconstitution.

The ability of adult or neonatal thymocyte populations separated by treatment with dilute anti-histocompatibility antisera and complement to influence the in vitro immune response of mouse spleen cells was studied. Inhibition or stimulation of the immune response was measured by the number of antibody forming cells appearing after 4 days of culture.

Major Findings:

1) The ability to respond to specific or nonspecific stimuli appears during late fetal life in both the BALB/c and (NZB x NZW)$F_1$ mouse thymus, and postnatally either decreases (MLR, PHA response) or increases (Con A, PWM responses), suggesting a differentiation into two functionally distinct subpopulations.

2) Thymic epithelial cells increase the functional activity of thymic lymphocytes dramatically, and lead to a homogenous population of cells which predominantly migrate to lymph nodes and have increased ability to induce graft-vs-host reactions.

3) Neonatal thymus contains cells which are capable of suppressing the immune response of adult spleen cells. Such suppressor cells are enriched by pretreatment with anti-H-2 serum, but their effect is reversed by neuraminidas

pretreatment.

Significance to Biomedical Research and the Program of the Institute:

T lymphocytes are crucial to cellular immune responses, antibody production and tolerance induction. The factors underlying their differentiation are not understood and knowledge of these areas would aid in immunologic reconstitution in immunologically deficient individuals.

Proposed Course of Project:

Attempts to direct in vitro differentiation of more primitive thymocytes using thymus epithelial cells will be made and further experiments aimed at an analysis of suppressor T lymphocyte function in the neonatal mouse are planned.

Project Subtitle III: Ontogeny of Bone Marrow Derived Lymphocytes

Project Description:

Objectives:

The development of B lymphocytes in mammals has been relatively difficult to study because no central lymphoid organ in which B cells develop has been identified in such animals. Moreover, in many mammalian species, a major degree of B cell development has been completed prior to birth, so that the study of B cell ontogeny is especially difficult in these animals. Fortunately, a substantial portion of mouse B lymphocyte differentiation appears to occur in the immediate post-natal period. This project subtitle is aimed at an exploration of the cellular events, occurring in the perinatal period, which are associated with the maturation of B lymphocytes.

Methods Employed:

Three surface markers associated with adult B lymphocytes were evaluated. Surface immunoglobulin (Ig) was detected with fluorescent and with $^{125}I$-labelled anti-Ig. Both an anti-K reagent and several anti-H chain reagents were employed. The receptor for aggregated immunoglobulin on B lymphocyte surfaces was identified by incubating the cells with heat aggregated guinea pig Ig, followed by rhodamine labelled rabbit anti guinea pig Ig. The complement receptor on B lymphocytes was evaluated by the formation of rosettes with sheep erythrocytes (E) coated with 19S anti-erythrocyte antibody (A) and complement (C).

Major Findings:

The spleen of the neonatal mouse contains few cells which bind anti-Ig antibody to their surface. Thus, Ig bearing cells constitute approximately 4% of the splenic lymphoid cells at birth. There is a marked elevation in

both the proportion and the absolute numbers of Ig bearing cells in the first several days of life. Indeed, by 24 hours of age, approximately 12 to 15% of splenic lymphoid cells bear surface Ig and there is a doubling in the total lymphocyte population. Thus, rapid and major changes in B lymphocytes occur in this early period. The expansion of splenic Ig bearing lymphocytes may occur either from the migration of differentiated B lymphocytes from a putative source organ into the spleen or from the development of Ig bearing lymphocytes from precursors already present within the spleen.

Neonatal spleen cells contain at least as high a percent of Ig bearing cells as do liver cells and other investigators have reported that the appearance of Ig bearing lymphocytes within the spleen precedes the appearance of these cells in the bone marrow. Consequently spleen and liver cell suspensions from neonatal mice were placed in culture and the number of Ig bearing cells present at 24 hours determined. A substantial increase ($> 2$ fold) in the frequency of these cells was observed during the culture period. This increase in frequency is not due to preferential survival of existing Ig bearing cells as the total cell yield is more than 80% of the cells placed in culture. These cells also express the receptor for aggregated Ig but not the complement receptor. Culturing the cells in the presence of mitomycin C diminished the number of Ig bearing cells found after 24 hour culture suggesting that the expansion requires cell division. Furthermore, pretreating neonatal spleen cell populations with anti-$\kappa$ and complement markedly reduces the number of Ig bearing cells found at 24 hours, indicating that the principal precursors of the Ig bearing cells found in 24 hour culture already have some surface Ig.

In the course of these studies of Ig bearing cells, it was noted that neonatal spleen cells failed to form rosettes with EAC indicating the absence of the complement receptor. Indeed, C57BL/6 mice have very few CRL in their spleen ($\sim 2\%$) even at 2 weeks of age when $\sim 30\%$ of their lymphocytes have surface Ig. This ratio of surface Ig bearing cells to CRL is markedly different from that in the adult spleen where $\sim 45\%$ of lymphocytes are Ig bearing and 25% to 30% bear the C receptor. Thus, there appears to be a substantial delay in at least one major step in B lymphocyte differentiation. Attempts are now in progress to determine if aquisition of immunocompetence on the part of B lymphocytes is related to the appearance of the complement receptor.

In addition, there is a clear strain dependence in the timing of appearance of the complement receptor. Thus, AKR mice, although having few CRL at 3 days of age, have substantial numbers at 1 week. At two weeks of age, more than 20% of the AKR spleen lymphocytes bear the C receptor. $F_1$ hybrids of AKR and C57BL/6 mice have frequencies of CRL similar to AKR suggesting this a dominant trait. At this time, backcrosses of $F_1$ to C57BL/6 mice are being undertaken to determine if this difference is controlled by a single gene.

Significance to Biomedical Research and the Program of the Institute:

These studies should lead to a fuller understanding of the mechanism of differentiation of the B lymphocytes. These cells are the precursors of antibody secreting cells and an understanding of their differentiation should allow intelligent approaches to the treatment of individuals with defects in B lymphocyte function.

Proposed Course of the Project:

These studies will be extended to more fully characterize the splenic cell which gives rise to immunoglobulin bearing lymphocytes. Attempts will be made to culture spleen and liver cells from prenatal mice in order to examine the appearance of an Ig bearing lymphocyte from a non-Ig bearing precursor. Studies on the significance of the delayed appearance of the complement receptor and of the genetics of this phenomenon will be expanded.

Project Subtitle IV: Regulation of B Lymphocyte Activation

Project Description:

Objectives:

The activation of immunocompent B lymphocytes into antibody secreting cells is a complex series of events generally involving participation of both macrophages and T lymphocytes. The analysis of the activation can be simplified by the use of antigens which cause B lymphocyte activation without (or with only limited degrees of) T lymphocyte participation. Others have already obtained important information through the analysis of responses to such "T-independent" antigens as type-III pneumococcal polysaccharide and polymerized flagellin. This approach is of considerable practical importance as such "T-independent" antigens are now used extensively as vaccines (e.g. pneumococcal and meningococcal polysaccharides).

Methods Employed:

This project was carried out in close collaboration with Dr. P.R.B. McMaster, Jr., LMI/NIAID, who prepared a DNP conjugate of Ficoll. Ficoll is a commercially available polymer of sucrose cross linked with epichlorhydrin. It has an average molecular weight of ~ 400,000. The conjugate was prepared by reacting ε-DNP-lysine with cyanogen bromide activated Ficoll. Immune responses and tolerance induction by DNP-Ficoll were evaluated in intact animals and in transfer studies by the production of serum anti-DNP antibody as determined by the binding of $^{3}$H-ε-DNP-L-lysine and by the appearance of antibody secreting cells in the spleen as determined by the formation of plaques using sheep erythrocytes coupled with DNP-β-alanyl-glycyl-glycine. These cells were provided through the courtesy of Drs. B. Merchant and J. Inman.

Major Findings:

Administration of 100 μg or more of DNP-Ficoll induces a profound state of immunologic tolerance. Such animals upon challenge with DNP hemocyanin in complete Freund's adjuvant produce little or no anti-DNP antibody although control animals synthesize large amounts of anti-DNP. Similarly, tolerance can be induced in primed cells, in transfer experiments. Thus, spleen cells from C57BL/6 mice primed to DNP-hemocyanin are transferred to irradiated syngeneic hosts. On the day of transfer, these animals receive 100 μg of DNP-Ficoll and 7 to 10 days later they are challenged with DNP-KLH. Under these conditions no anti-DNP antibody is produced although similar animals which had not received DNP-Ficoll secrete substantial amounts of anti-DNP antibody.

Smaller doses of DNP-Ficoll induce a primary immune response. Thus, the injection of C57BL/6 or AKR mice with 1 or 10 μg of DNP-Ficoll results in substantial numbers (10,000 or more) splenic DNP-specific plaque forming cells 4 days later. This response is largely IgM in character and occurs without the use of adjuvants. Animals which have received an initial immunization with DNP-Ficoll do not demonstrate a secondary response when rechallenged 30 days layer. Finally, both congenitally athymic mice (nu/nu) and mice which have been thymectomized as adults, irradiated and restored only with bone marrow (TxBM) mount responses to DNP-Ficoll which are equivalent to those of normal mice.

Thus, DNP-Ficoll is a thymus independent antigen which initiates primary IgM responses when an immunogenic dose is used, does not induce a state of immunologic memory and, if large doses are used, induces specific immunologic tolerance in both unprimed and primed animals. This compound thus appears to be an excellent model for the immunogenic polysaccharides which are used as vaccines in humans. In particular, it now appears that most adult humans become primed to antigens cross reactive with specific bacterial polysaccharides by experience with microorganisms and that intentional immunization of adults is, effectively, an attempt to induce a secondary response. An appropriate model to study the regulation of such secondary responses to T-independent antigens would be valuable.

Spleen cells from C57BL/6 mice primed to DNP-hemocyanin were transferred to irradiated recipients who were challenged with either nothing, an immunogenic dose of DNP-Ficoll (10 μg) or DNP-KLH (100 μg). Mice which had been challenged with DNP-Ficoll developed substantial numbers of IgG DNP-specific plaque forming cells. Indeed, such animals had equivalent numbers to those seen after challenge with DNP-KLH although their serum anti-DNP titers were lower. Thus, in primed animals, a "T-independent" antigen may induce a IgG antibody response whereas the response in normal animals is largely IgM. Attempts are now in progress to evaluate the role of T lymphocytes in these responses and to determine the characteristics of the B lymphocytes which are the predecessors of the anti-DNP antibody secreting cells in this system.

Significance to Biomedical Research and to the Program of the Institute:

The detailed analysis of responsiveness to and tolerance induction by DNP-Ficoll will allow a description of the control of activation of B lymphocytes. In particular, this will provide an excellent model system for responses of humans to bacterial polysaccharides used as vaccines.

Proposed Course of the Project:

An analysis of the role of T lymphocytes in the activation of IgG anti-DNP secondary responses by DNP-Ficoll, will be made and a study of expansion of memory cells under these conditions is planned. Furthermore, attempts to characterize the class of surface Ig on cells responding to DNP-Ficoll in this sytem will be undertaken.

Publications:

Janeway, C.A., Jr. and Paul, W.E.: Hapten-specific augmentation of the anti-idiotype antibody response to hapten-myeloma protein conjugates in mice. Eur. J. Immunol., in press.

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Stobo, J.D., Talal, N. and Paul, W.E.: Lymphocyte classes in New Zealand mice. II. Decreased frequency of immunoglobulin bearing lymphocytes and increased frequency of lymphocytes lacking detectable theta or immunoglobulin determinants. J. Immunol. 109: 701, 1972.

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Stobo, J.D. and Paul, W.E.: Functional heterogeneity of murine lymphoid cells. III. Differential responsiveness of "T" cells to phytohemagglutinin and concanavalin A as a probe for "T" cell subsets. J. Immunol. 110: 362, 1973.

Stobo, J.D.: Phytohemagglutinin and concanavalin A: Probes for murine "T" cell activation. Transplant. Rev. 11: 60, 1972.

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Stobo, J.D., Rosenthal, A.S. and Paul, W.E.: Functional heterogeneity of murine lymphoid cells. V. Lymphocytes lacking detectable surface θ or immunoglobulin determinants. J. Exp. Med., in press.

Yoshida, T., Janeway, C.A. Jr. and Paul, W.E.: Activity of migration inhibitory factor in the absence of antigen. J. Immunol. 109: 201, 1972.

Serial No. NIAID - 142
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Genetic Control of Immune Response

Previous Serial No.: Same

Principal Investigators: Dr. Ethan Shevach and Dr. Ira Green

Other Investigators: Project Subtitle I. Dr. William E. Paul, LI/NIAID
Dr. Schlomo Ben-Sasson, LI/NIAID
Dr. Alan S. Rosenthal, LCI/NIAID
Project Subtitle II. Dr. Irwin Scher

Cooperating Units: U.S. Naval Medical Center
Bethesda, Maryland

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 3 | | | 3 |
| Professional | 1 1/2 | | | 1 1/2 |
| Other | 1 1/2 | | | 1 1/2 |

Project Subtitle I: Effect of Anti-histocompatibility Antisera on Immune Response Gene Function

Project Description:

A series of genes have been described in guinea pigs, mice, rats and, recently, in man which control the capacity to mount specific immune responses. A number of such immune response (IR) genes have been shown to be linked to the major histocompatibility (H) antigens of the species and to function primarily in the T lymphocytes. Alloantisera prepared by cross immunizing strain 2 and strain 13 guinea pigs markedly inhibit the in vitro proliferative response to antigens the response to which is controlled by an IR gene which is linked to the histocompatibility antigen against which the serum is directed. It is therefore likely that the immune response gene product is present on the surface of the T lymphocyte. Experiments were designed to investigate the mechanism by which alloantisera which are primarily directed against H antigens can block the function of the IR gene product and to determine whether T lymphocyte function other than in vitro proliferation can also be blocked.

Although the in vitro proliferative response to antigen is primarily a property of the T lymphocyte, it is clear that in the guinea pig this response requires the presence of macrophages which play a role in the initial binding, handling, and processing of the antigen. It is possible that the alloantisera inhibit the in vitro proliferative response by acting on the macrophage rather than on the T lymphocyte. Experiments were therefore designed to define the specific cell type on which the alloantisera act and to define the role of the macrophage in the regulation of genetic control of the immune response.

As a corollary of these studies, it was noted that it was difficult to prepare alloantisera which killed greater than 40% of lymph node cells; this suggested that the alloantisera killed a specific subpopulation of lymphoid cells. It was therefore of interest to determine the distribution and quantity of H antigen on the different lymphocyte populations in the guinea pig.

Major Findings:

In order to determine whether the alloantisera were directed solely against H antigens or whether they also contained antibodies against the IR gene product, the 13 anti-2 serum was absorbed with a large number of malignant B lymphoid cells, the $L_2C$ leukemia cell. This leukemia cell is rich in H antigen and, because it is of B cell lineage, may not bear the IR gene product on its surface. In most experiments the absorbed anti-2 serum, which was devoid of any cytotoxic antibodies to strain 2 H antigens, also failed to inhibit the in vitro proliferative response of $(2x13)F_1$ cells to DNP-GL whereas unabsorbed sera completely inhibited the response to DNP-GL. This suggests that the alloantisera are solely directed against H antigens and absorption with cells which contain only H antigens on their surface removes their inhibitory activity; alternatively, the $L_2C$ cell, although it is a B cell, might also bear the IR gene product on its surface and thus absorb anti-IR antibodies.

Another approach to determining the specificity of the alloantisera was to test their function in the inhibition of IR gene function in a situation where an appropriate IR gene is not linked to H antigen to which it is linked in inbred populations. In inbred guinea pigs and some Hartley animals, the IR gene which controls responsiveness to the copolymer of L-glutamic acid and L-alanine (GA) is linked to genes which control responsiveness to DNP-GL and to strain 2 H antigen genes; however, in other Hartley animals, the GA gene is not linked to either the DNP-GL gene or to strain 2 H genes. It was therefore possible to test whether the anti-2 serum would inhibit GA gene function in the two disparate populations of GA responsive Hartley guinea pigs. The results demonstrated that the in vitro proliferative response to GA could be inhibited by the anti-2 serum only in cultures from animals that were $GA^+$, $DNP\text{-}GL^+$, $2^+$. Cultures derived from animals that were $GA^+$, $DNP\text{-}GL^-$, $2^-$ were not inhibitable. This result suggests that the anti-2 serum is directed solely against 2 H antigen specificities and can only inhibit the GA gene product when it is linked to strain 2 H antigen specificities. It was also possible to prepare antisera in strain 13 animals against lymphocytes from the $GA^+$, $DNP\text{-}GL^+$, $2^-$ Hartleys. The ability of these antisera to inhibit the GA response in $(2x13)F_1$ animals immune to DNP-GL and GA was tested. Only the antisera which had been prepared against the $GA^+$, $DNP\text{-}GL^+$, $2^+$ Hartley animals

could inhibit both the DNP-GL response and the GA response. The 13 anti-$GA^+$. $DNP\text{-}GL^-$, $2^-$ sera were devoid of inhibitory activity. It was therefore concluded that in this experimental system the alloantisera act by blocking histocompatibility antigen and sterically interfering with the IR gene product which is closely linked to H antigen on the cell surface. It has so far been impossible to prepare antisera which are solely directed against the IR gene product. This result suggests that IR gene products and H antigens are closely linked on the cell surface and are consistent with the concept that they may be physically linked to each other.

Thus far, the only T lymphocyte function for which alloantisera inhibition linked to IR gene function has been demonstrated is in vitro DNA synthesis. As it now appears likely that different subpopulations of T lymphocytes with distinct functional properties exist, it was felt that an investigation of the capacity of alloantisera to inhibit other T lymphocyte functions would be valuable. The production of migration inhibitory factor is a major effector function of T lymphocytes and appears to be of importance in the development of delayed hypersensitivity skin reactions. Furthermore, it has been proposed that the T cells mediating in vitro DNA synthesis and MIF production may be distinct. Thus, the capacity of anti-13 and anti-2 alloantisera to inhibit MIF production by immune $(2x13)F_1$ peritoneal exudate lymphocytes was tested. In these studies, DNP-GL, DNP-guinea pig albumin (GPA) and the copolymer of glutamic acid and tyrosine (GT) were employed. The response to DNP-GL is controlled by an IR gene linked to strain 2 H genes while responses to both DNP-GPA and GT are controlled by genes linked to strain 13 H genes. It was shown that the capacity of lymphocytes from immune $(2x13)F_1$ animals to produce MIF in response to DNP-GL was inhibitable by anti-2 serum but not anti-13 serum while the production of MIF in response to either DNP-GPA or GT was inhibited by anti-13 serum but not by anti-2 serum. This result suggests that the capacity of alloantiserum to inhibit IR gene function is not limited to a single function or type of T lymphocyte.

Although it is clear that alloantisera inhibit T lymphocyte function, it has not been shown whether the serum function by interacting with the T lymphocyte itself, the macrophage (which presents antigen to the T cell) or both cell types. In order to precisely determine the population of cells on which the alloantisera act, strain 13 macrophages were pulsed with DNP-GL and added to strain 2 T lymphocytes. In this situation the 13 anti-2 serum should only inhibit the proliferative response to DNP-GL if it acts on the T lymphocyte. However, it was noted that strain 13 macrophage associated antigen was ineffective in activating strain 2 lymphocytes. In order for macrophages to activate T lymphocytes to proliferate in vitro both cell types must be histocompatible. Semi-allogeneic macrophages and T cells do cooperate and it was possible to examine the situation when 13 macrophages were pulsed with DNP-GL and added to F-1 lymphocytes. A number of important results were obtained from this experiment: the F-1 T cells by 13 macrophages pulsed with DNP-GL was very inefficient when compared to the ability of strain 2 or F-1 macrophages to carry out this process. The stimulation of F-1 cells by 13 macrophages pulsed with DNP-GL could be completely blocked by the 13 anti-2 serum. This suggests that the alloantisera act on the T lymphocyte rather than on the macrophage. However, this same stimulation could also be blocked by the 2 anti-13 serum

which suggests that the alloantisera can also inhibit lymphocyte-macrophage cooperation. The inefficient activation of F-1 T cells by strain 13 macrophages pulsed with DNP-GL is best explained by postulating that the macrophage must bind to the lymphocyte at or near the appropriate H antigen. Since the DNP-GL IR gene product is physically closely related to 2 H antigen, the 13 macrophage cannot effectively present the DNP-GL antigen to its antigen receptor site on the T lymphocyte. These results suggest an important role for the macrophage in the regulation of antigen recognition by the T lymphocyte; they also suggest that the effect in the "non-responder" animals is probably not at the level of macrophage antigen processing step, but rather at the T lymphocyte antigen recognition step.

Allo antisera prepared by cross-immunizing strain 2 and strain 13 animals kill only 30-40% of lymph node cells and those cells that remain alive after killing by the alloantisera are markedly depleted in immunoglobulin bearing B cells. Absorption studies of the anti-2 serum demonstrated that lymph node cells contain 7 to 8 times as much alloantigen as thymocytes, while pure B lymph node cells contain about 2 to 3 times the amount of alloantigen as T lymphocytes. It was also shown that those lymph node cells which remain alive after treatment with the alloantisera and complement fail to respond to endotoxin, a B cell mitogen, but respond normally to PHA, a T cell mitogen. These studies demonstrate an increased content of H antigen on the B lymphocyte compared to the T lymphocyte. Since H antigens play a role in the cooperation between macrophages and T cells and between B cells and T cells, information as to the distribution of H antigens on these cells may have important implications for cell to cell interaction in the immune response.

Significance to Biomedical Research and the Program of the Institute:

These studies have demonstrated that both the IR gene product and histocompatibility antigens play an important role in the mechanism of antigenic recognition by the T lymphocyte. The chemical nature of the antigen receptor and the mode of action of this receptor on the T lymphocyte are unknown at the present time. It is likely that further study and characterization of the IR gene product will aid in the elucidation of the mechanism of T lymphocyte function in the regulation of the normal immune response. In addition, since susceptibility to a number of malignant and auto-immune diseases has been linked to histocompatibility type, IR genes may play an important role in susceptibility to a variety of diseases.

Proposed Course of Project:

These studies will be further pursued with a view to establishing whether alloantisera block the binding of antigen to specific receptors on T lymphocytes or block the activation of the cell after the binding of antigen. Furthermore, definitive tests of the possibility that anti-IR antibodies exist are planned. Attempts to chemically characterize the IR gene product will also be undertaken.

Project Subtitle II: Study of the Immune Response in Humans to a Terpolymer of Glutamic Acid Lysine and Tyrosine (GLT)

Project Description:

Objectives:

The ability of guinea pigs, mice and rats to immunologically respond to simple synthetic poly amino acid antigens has been shown to be controlled by a number of different dominant autosomal genes (IR genes). In mice and guinea pigs these IR genes are linked to but are not identical with genes controlling histocompatibility (H) antigens.

In humans the frequency of occurrence of a variety of diseases has also been associated with the presence of certain H antigens.

The diseases in which such associations have been noted include: Hodgkins disease, acute lymphatic leukemia, lupus erythematosus, gluten sensitive enteropathy, psoriasis, dermatitis herpetiformis and ankylosing spondylitis. In addition, the ability of certain individuals to make an anti IgE response to a minor pollen antigen Ra-5 has been linked to the presence of HL-A7.

Since the link between the increased frequency of certain HLA types in certain human diseases may be due in turn to the linkage of IR genes in humans to certain HLA types, it is of importance to establish the existence of H linked Ir genes in humans and to develop methods for the study of the functions of such genes. Therefore, humans were immunized with the random terpolymer of L-glutamic acid, L-lysine and L-tyrosine (GLT) and the presence or absence of an immune response to this antigen was correlated with their HL-A type.

Methods Employed:

The simple synthetic antigen GLT was chosen for these studies because the demonstration of IR genes is made simpler when an antigen carries only a limited number of structures which are potentially recognizable by Ir gene products.

Human volunteers were injected intradermally with 50 µg GLT in saline; the injections were performed at weekly intervals. Delayed hypersensitivity skin reactions (D.H.) were evaluated at the second and third injection of GLT. Antibody was measured by Farr assay using $^{125}I$ GLT and also by passive hemagglutination using sheep erythrocytes coated with GLT. Lymphocytes were obtained from peripheral blood and the degree of lymphocyte transformation after exposure of lymphocytes to GLT in vitro was assayed by standard techniques. The HL-A type of the individual was determined by the Terasaki micro-cytotoxicity technique.

Major Findings:

Twenty-two adults were immunized with GLT. Ten of the individuals demonstrated significant D.H. reactions (greater than 7 mm in diameter). Some of these D.H. reactions reached 30 mm in diameter. Twelve individuals had either no D.H. reactions or small reactions (smaller than 4 mm in diameter). Only 1 of 22 individuals demonstrated a reaction at the site of the first injection of GLT and that was minor. No antibody to GLT could be demonstrated by either Farr assay or hemagglutination in any individual regardless of the size of their D.H. reaction. Furthermore, no correlation was observed between the degree of in vitro lymphocyte transformation in response to GLT and the degree of D.H. observed in the donor of the lymphocytes. However, lymphocytes from unimmunized individuals never demonstrated transformation in vitro in response to GLT.

All 22 patients were HL-A typed. Nine of ten patients with large D.H. reactions to GLT possessed the HL-A2 antigen whereas among 12 individuals who had small or no D.H. reactions to GLT only 3 had the HL-A2 antigen. More patients are being immunized with GLT to see if this relationship between responsiveness and the presence of the HL-A2 antigen will be maintained. Attempts to develop other methods of evaluating responsiveness are also in progress.

Significance to Biomedical Research and the Program of the Institute:

The identification of HL-A linked IR genes in man and an understanding of their mechanism of function may eventually aid in the understanding of the relationship between the frequency of certain HL-A types and certain human diseases and in turn will aid in understanding the etiology of these diseases.

Proposed Course of Project:

More patients will be immunized with GLT. In addition new synthetic polyamino acid antigens of either simpler or more complex structure will be used. When several IR genes have been identified in humans, individuals with specific diseases can be immunized with synthetic antigens to see if they possess certain of these IR genes. Thus the presence or absence of certain IR genes may eventually be linked to the occurrence of certain diseases.

Publications:

Green, I., Paul W.E. and Benacerraf, B.: Histocompatibility linked genetic control of the immune response to hapten guinea pig albumin conjugates in inbred guinea pigs. J. Immunol. 109: 457, 1972.

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1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Lymphocyte Interactions, Receptors and Functions

Previous Serial No.: Same

Principal Investigator: Ira Green

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Cooperating Units: None

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 4 1/2 | | | 4 1/2 |
| Professional | 3 | | | 3 |
| Other | 1 1/2 | | | 1 1/2 |

Project Subtitle I: Receptors for Antigen-Antibody and Antigen-Antibody Complement Complexes on Lymphoid Cells

Project Description:

Objectives:

Cells involved in the immune response can be functionally characterized by the presence of different membrane antigens and receptors. In the mouse, T cells can be identified by the presence of the θ isoantigen; in man, T cells have been found to form rosettes with sheep red blood cells (E). B cells, in most species studied, can be identified by the presence of easily detectable surface immunoglobulin and a receptor for antigen-antibody-complement complexes (EAC). Monocytes and macrophages, the third cell type involved in the immune response, also bear a receptor for EAC, but they can be distinguished from B cells because they also bear a receptor for antigen-antibody complexes (EA). The presence or absence of these various receptors on cells of unknown type may provide information as to their origin. In this study, malignant lymphoid cells from both mice and humans were examined for the presence of these various receptors.

Methods Employed:

In order to detect the EAC receptor, the lymphoid cells to be studied are incubated with red cells that have been coated with anti-red cell antibody of the IgM type and mouse serum as the source of complement. The percentage of cells binding these complexes is determined under the microscope. The test may be performed on lymphoid cells which have been disassociated in a suspension or on frozen sections of lymphoid cells from spleen or lymph nodes. The EA receptor is detected in a similar fashion with red cells coated with anti-red cell antibody of the IgG type. The E binding human T cells can only be detected on lymphoid cells in suspension. The mouse leukemias and lymphomas were also examined for the presence of the θ isoantigen and surface immunoglobulin by standard immunofluorescent techniques.

Major Findings:

All of the mouse leukemias and lymphomas studied arose spontaneously in one strain of mice (BALB/c) and all have been passed serially for several generations. All of the tumors studied can be assigned to one of the classes of normal murine lymphocytes. Thus, of the 20 lymphomas, 10 were found to bear the θ isoantigen and, thus, were of T cell type; 6/20 tumors had surface Ig of the IgM class and are classed as B cell in origin. It should be noted that the receptor for EAC could not be found on any of these tumors. 4/20 lymphomas bore the receptor for EA and thus appear to be of monocyte origin.

The studies on human leukemias and lymphomas have concentrated on determining the origin of the cell type in solid lymphoid tumors such as Hodgkin's disease and lymphosarcoma. The areas in spleen or lymph node that bind EAC or EA are determined and the same section is then examined for the presence of malignant lymphoid cells. Malignant cells in two patients with lymphosarcoma have both been shown to bind EAC. This suggests that these malignancies

are of B cell origin. One case of histiocytic medullary reticulosis and one case of leukemic reticuloendotheliosis were studied. In both, cells in lesion bound EA suggesting they were of monocyte origin. Cells from patients with nodular (Giant follicle) lymphoma both bound EAC and are, thus, B cell origin.

Significance to Biomedical Research and the Program of the Institute:

The presence of the receptors for EAC, EA and E, and of surface immunoglobulin on lymphoid or monocytoid cells of unknown type provides an easily detectable clue as to their origin. The ability to classify lymphoid tumors as to their cell type may not only be of theoretic interest, but may also lead to therapeutic advances. Furthermore, the availability of large numbers of malignant cells with receptors characteristic of normal cells offers the opportunity for detailed study and characterization of these receptors. In addition, some of the murine lymphomas have been shown to have some of the immunologic functions of the cell type from which they originated.

Proposed Course of Project:

The studies on human malignant lymphomas and leukemias are being continued and expanded.

Project Subtitle II: Precise Identification of the Types of Mononuclear Cells Infiltrating the Tissues in Various Human Pathological Conditions

Project Description:

Objectives:

Different types of lymphocytes and monocytes are observed in tissue lesions in a variety of pathological states. The precise reason why these mononuclear cells are present and their role in the pathological processes remains obscure.

Methods Employed:

Recent technological advances allow for the identification of human monocytes and bone marrow derived (B) lymphocytes in tissue sections by virtue of the presence of specialized surface receptors on these cells. As summarized in Subtitle I, B lymphocytes have a receptor for the third component of complement (C3) and monocytes have receptors for C3 as well as for immunoglobulin G (IgG). Red cells coated with either C3 or IgG serve as indicator particles capable of detecting cells in unfixed frozen sections which bear these receptors.

Major Findings:

Patients with dermatological diseases were chosen for study first because

of the ready accessibility of the involved tissues and the frequency of mononuclear cell infiltration. Three patients were studied. First, a patient with chronic lymphatic leukemia (CLL) with a skin nodule. The mononuclear cells in the section bound only sheep erythrocytes coated with IgM and C (19S EAC) indicating that the infiltrate was composed of B lymphocytes. This, of course, was not unexpected since CLL is a disorder of B lymphocytes. The second patient had an intraepithelial squamous cell carcinoma with a dense infiltrate of small lymphocytes. These lymphocytes in sections failed to bind 19S, EAC or red cells coated with IgG anti-erythrocyte antibody (7S EA). These cells thus appear to be T lymphocytes. Furthermore, cells teased from these lesions were killed in a $^{51}Cr$ release technique by a heterologous antiserum specific for T cells. The third patient was a child with acute leukemia who developed a severe GVH reaction following an allogeneic bone marrow graft. Sections of the exfoliative skin lesions from this patient contained cells almost all of which bound only 7S EA. Thus they were monocytes.

Another human disease associated with lymphocytic infiltrates is Sjögren's Syndrome (SS). Here lymphocytes are seen in the salivary and lacrimal glands and severe destruction of the paranchema of these organs is observed. Sections of lip biopsies containing minor salivary glands were examined in three patients with SS using these techniques. In the biopsies from two of the patients almost all of the mononuclear cells infiltrate bound 19S EAC and were therefore B cells. In the third patient 19S EAC were bound only to the central portion of the infiltrate. The peripheral lymphoid cells failed to bind 19S EAC or 7S EA. These lymphocytes are probably T lymphocytes.

Significance to Biomedical Research and Programs of the Institute:

During the last 12 years a variety of *in vitro* studies have demonstrated that T lymphocytes, B lymphocytes and monocytes can produce damage to target cells by a variety of mechanisms. However, no real synthesis between the *in vitro* phenomenology of target cell killing and *in vivo* pathological events has been made. The identification and exact distribution of the various types of mononuclear cells in pathological tissues is a first step in this direction.

Proposed Course of Project:

More disease states in which lymphatic infiltrates are present will be investigated with these techniques. Eventually mononuclear cells will be teased out of pathological infiltrates and their cytotoxicity assayed *in vitro* against appropriate target cells. By this means the role of the infiltrating mononuclear cell in the pathological situation can be established.

Project Subtitle III: Surface Immunoglobulin on Lymphocytes of Normal Humans and of Patients with Sjögrens Syndrome

Project Description:

Objectives:

Peripheral blood lymphocytes of humans consist of at least two major classes, the thymus derived (T) and bone marrow derived (B). These cells bear surface markers (discussed in the previous subtitles) which allow their identification. In particular, many of the B lymphocytes bear surface immunoglobulin and an investigation of the number of such cells and of the type of immunoglobulin present on their surface in normal and patients with Sjögren's syndrome, an autoimmune disease with a propensity to lymphoproliferation, has been studied.

Methods Employed:

In addition to the various tests described in the previous subtitles, the binding to lymphocyte surfaces of antisera specific for various immunoglobulin heavy (H) chain classes was carried out and the capacity of aggregated guinea pig immunoglobulin to bind to lymphocytes was determined. The latter test measures B lymphocytes by virtue of their receptor for aggregated immunoglobulin.

Major Findings:

In normal individuals lymphocytes bearing each of the major H chain classes were found. The frequency of cells bearing $\gamma$G was 7.5 ± 1.8, $\gamma$M 6.2 ± 1.0, $\gamma$A 7.0 ± 1.3 and $\gamma$D 5.5 ± 0.5. The frequency of $\gamma$D bearing cells is especially striking in view of the way low serum concentrations of IgD. This finding was reported in last years annual report. Further tests demonstrating the specificity of this finding including absorption of antisera on $\gamma$D-sepharose and preincubation of cells in non-fluorescent anti-$\gamma$D sera have been performed. Furthermore, double label experiments have shown that all lymphocytes with surface $\gamma$D are B cells. Thus, all $\gamma$D bearing cells bind aggregated guinea pig immunoglobulin and no E rosette forming cells have surface IgD.

Seven patients with well established Sjögren's syndrome were evaluated for lymphocyte surface Ig. In general, these patients have moderate elevations in the proportion of B lymphocytes in peripheral blood and little chang in the frequency of T cells. However, there were striking (~ 3-fold) elevations in the frequency of cells bearing Ig of the G, A, M and D classes in several of these patients. Double labeling experiments showd that surface Ig was limited to B lymphocytes. Thus, it appears that individual lymphocyte bearing multiple classes of immunoglobulin are present in the blood of some patients with Sjögren's syndrome.

Significance to Biomedical Research and Programs of the Institute:

The present of substantial numbers of IgD bearing lymphocytes in the blood of normal people coupled with exceedingly low serum concentrations is of considerable significance. The study of variability of these cells in disease states may provide a clue to the function of IgD. At the current time there is no known biological function for this class of Ig. The presence

of several classes of Ig on individual B lymphocytes from patients with Sjögren's syndrome is puzzling but may provide important clues to the pathogensis of this disease and, in particular, to the lymphoproliferative disorders associated with this syndrome.

Proposed Course of Project:

The prime goal of further study will be to delineate whether IgD is a produce of the lymphocyte on which it is found or whether it attaches to the cell through a cytophilic mechanism. In regard to the study of Sjögren's syndrome, attempts will be made to correlate lymphocyte surface Ig with clinical status and to determine whether individual B cells actually synthesize immunoglobulins of more than a single class.

Project Subtitle IV: Antibody-Dependent Lymphoid-Cell Mediated Cytotoxicity

Project Description:

Objectives:

Lymphoid cells from unsensitized donors have the capacity to destroy antibody coated target cells in vitro. The aim of these studies is to determine 1) the precise identity of the effector cell, 2) the mechanism of target cell destruction including the role of complement, and the nature of the effector cell surface receptors, and 3) the biological role of this reaction.

Methods Employed:

Target cell destruction is evaluated by the release of $^{51}Cr$ from antibody-coated burrow erythrocytes after 24 hour incubation with effector lymphoid cells in culture.

(1) Effector cell type and nature of its surface receptor for the target cell.

Heat aggregated guinea pig gammaglobulin binds to the Fc receptor on B lymphocytes and indirect immunofluorescence was used to determine the frequency of aggregate-bearing cells in mouse spleen, lymph node and thymus. Total B cell frequency in these cell populations was determined by staining with fluorescent anti-mouse kappa chain antiserum. These aggregates as well as soluble antigen-antibody complexes were tested for their inhibitory effect on the cytotoxic reactions.

(2) Role of complement

Lymphoid cells from guinea pigs, mice and rabbits genetically deficient in the 4th, 5th and 6th C components, respectively, were tested for their ability to mediate antibody-dependent cytotoxicity. In each case the system was totally free of the respective C component: this was accomplished by using serum from the C deficient animals to support the cultures instead of fetal calf serum, and using anti-target cell antiserum raised in such C

deficient animals. In addition, guinea pig serumdepleted of C3 by in vivo cobra venom treatment was used to support the culture medium instead of FCS in experiments to test the requirement for C3. Furthermore, the capacity of an excess of C coated erythrocytes to inhibit cytotoxicity was tested in order to determine the role of the C receptor on the effector cell.

Major Findings:

(1) Effector cell type and its receptor

We have previously reported that T lymphocytes are not required for antibody-dependent lymphoid cell mediated cytotoxicity. Furthermore, we have subsequently reported that cells which do mediate this reaction have a C receptor and appear to be members of the B lymphocyte class. However, the data suggested that only a relatively small subset of these B cells had a role in the cytotoxic reaction.

A subpopulation of lymphocytes in spleen and lymph nodes were found to bind aggregated γ-globulin by immunofluorescent staining. When aggregated γ-globulin or soluble complexes were added to the cultures, cytotoxicity was markedly inhibited, strongly suggesting that aggregate binding cells are the effector cells and that aggregates inhibit by competing for the aggregate receptor on the effector cell and, indeed, that this aggregate receptor is the one used by the killer cell in its reaction with the antibody target cell complex. Moreover, we noted a striking paucity of aggregate binding cells in the lymph node as compared to the spleen, and also that the number of aggregate binding cells was much less than the number of Ig-bearing cells in the lymph node. These findings are consistent with our report of the greatly diminished cytotoxicity of mouse lymph node cells as compared to spleen cells.

(2) Role of complement:

Cytotoxicity was not diminished in the in vitro assay in the complete absence of the 4th, 5th or 6th C components. This indicates that neither the classical nor the alternate pathways have a role in this reaction. In addition, the evidence is against an as yet underscribed pathway activating C3 since cytotoxicity was not diminished in the absence of C3. Finally, an excess of C coated sheep red cells did not inhibit cytotoxicity. This strongly suggests that the C receptor on the effector cell does not have a role in cytotoxicity in the in vitro system as the C coated sheep red cells would otherwise be expected to inhibit the reaction by competing for this receptor.

Significance to Research and the Program of the Institute:

There is increasing evidence that antibody-dependent lymphoid cell mediated cytotoxicity may have a major biological role in a variety of pathological tissue damage. Knowledge of the basic mechanisms of this reaction in vitro may lead to its further elucidation in vivo and may provide

the means to its manipulation in disease states.

Proposed Course of the Project:

Further attempts to characterize the B lymphocyte subclass causing cell damage and to determine the molecular basis of target cell lysis will be made. In addition, attempts to relate this mechanism to pathogenesis of disease will be extended.

Project Subtitle V: Proliferation of Bone Marrow-Derived Lymphocytes In Vitro In response to Stimulation by Antigen, Mitogens and Anti-Immunoglobulin Antisera

Project Description:

Objectives:

Until recently, the proliferative response of lymphocytes in vitro to specific antigens and the non-specific mitogens phytohemagglutinin (PHA) and Concanavalin A (Con A) was believed to be solely a property of thymus-derived lymphocytes (T cells). Furthermore, the proliferative response to pokeweed mitogen (PWM) was felt to be predominantly a property of bone marrow-derived lymphocytes (B cells). Recently, bacterial endotoxin lipopolysaccharide (LPS) was described as a B cell stimulant exclusively. And finally the proliferative response of lymphocytes to anti-immunoglobulin antisera was believed to be solely a property of B cells. However, the experiments which led to these conclusions were performed using pure populations of either T cells or B cells prepared prior to putting the cells in culture with antigen, mitogen or anti-immunoglobulin antisera.

The recent discovery that B cells bear a receptor for activated third component of complement (C3) has enabled us to identify B cells in mixed T and B cell populations even after a period of culture. By mixing antibody and complement coated sheep erythrocytes (19S EAC) with lymphoid cells, rosettes form around complement receptor lymphocytes (CRLs) which are B cells.

The proliferative response of guinea pig lymph node cells (GPLNC), mouse spleen cells (MSPL) and rabbit peripheral blood lymphocytes (RPBL) to specific antigens, PHA, Con A, PWM, LPS and anti-immunoglobulin antisera has been examined. Briefly, the technique employed involves the simultaneous examination of lymphocytes that had been cultured for 48 hours for the presence of CRLs and for incorporation of tritiated-thymidine ($^{3}$H-T) into these CRLs. This allows the analysis of the proliferative response of mixed T and B cell populations after the period of culture and directly determines the contributions of B cells to the total proliferative response.

Methods Employed:

Briefly, the method entails culturing GPLNC, MSPL and RPBL from immunized

animals or from non-immunized animals in complete medium supplemented with heat inactivated serum with or without optimal concentrations of specific antigen, PHA, Con A, PWM, LPS or anti-immunoglobulin antisera. The immunizing antigens were ovalbumin in guinea pigs, inactivated vaccinia virus in rabbits, and keyhole limpet hemocyanin in mice. Anti-immunoglobulin antisera were raised in rabbits: rabbit anti-guinea pig immunoglobulin (RaGPIg), rabbit anti-rabbit allotype (RaRA), and rabbit anti-mouse kappa chain (RaMK). Eighteen hours prior to harvesting the cultures, one microcurie of $^{3}$H-T was added to each culture tube. Lymphocyte incorporation of $^{3}$H-T was determined for three replicate tubes. The cells in the remaining three replicate tubes were pooled, washed, and mixed with 19S EAC to form CRL rosettes. The resulting cell suspensions were processed for radioautography. Radioautographs were examined simultaneously for total number of mononuclear cells, total number of rosetted cells, total number of grained cells, and total number of simultaneously grained and rosetted cells.

Major Findings:

1) As measured by cpm of $^{3}$H-T incorporated into cultured lymphocytes and percentage of grained mononuclear cells (from radioautographs), specific antigens, PHA, Con A, PWM, LPS and anti-immunoglobulin antisera stimulated GPLNC, MSPL and RPBL to proliferate except in the following two cases. LPS failed to stimulate RPBL and RaMK failed to stimulate MSPL.

2) The percentage of CRL present after 48 hours in vitro was virtually the same for stimulated and non-stimulated cultures.

3) In all cases studied by the CRL rosette radioautography technique, the fraction of proliferating lymphocytes that were CRLs was substantial. Specifically in unstimulated cultures the percentage of proliferating lymphocytes that were CRLs varied from ~10 to 50%. In antigen stimulated cultures, the percentage ranged from ~15 to 45%. In PHA, Con A and PWM stimulated cultures, the percentage ranged from ~10 to 30%. For anti-immunoglobulin stimulated GPLNC and RPBL, the percentage was ~70 to 90%. And for LPS stimulated GPLNC, the percentage was 83% whereas for LPS stimulated MSPL the percentage was 26%.

4) For all stimulants studied by the CRL-rosette radioautography technique, the fraction of CRLs that were proliferating was significantly increased.

Significance to Biomedical Research and the Program of the Institute and Future Course of the Project:

Since it has been shown that neither stimulated or unstimulated T cells acquire a C3 receptor in vitro and since all CRL bear surface immunoglobulin. it can be concluded that B cells participate to a significant degree in in vitro proliferative responses. Antigen, PHA and Con A stimulation causes B

cell proliferation as well as T cell proliferation. PWM induces T cell proliferation predominantly but stimulates B cells as well. LPS stimulates B cells predominantly in GPLNC but T cells appears to stimulate as well as B cells in MSPL. Anti-immunoglobulin initiates B cell proliferation exclusively in GPLNC and RPBL. Because of these findings, the characterization of lymphocyte responses as being either solely T cell or solely B cell in nature must be revaluated. Indeed, in mixed cell populations both T cells and B cells respond to most stimulants.

These findings do not, however, establish the B cells how the B cells are activated by mitogens the response to which requires the presence of T lymphocytes. Two major mechanisms must be considered, 1) indirect stimulation of B cells by T cells bearing stimulant in a specific spatial array, 2) indirect stimulation of B cells by T cells producing non-specific blastogenic mediators. Experiments attempting to elucidate these mechanisms are currently in progress.

The methodology employed in these studies may be used to evaluate human lymphocyte proliferative responses in malignant, auto-immune, and immunodeficiency disorders.

Publications:

Shevach, E.M., Stobo, J.D. and Green, I.: Immunoglobulins and theta bearing murine leukemias and lymphomas. J. Immunol. 108: 1146, 1972.

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PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Cellular Basis of Antibody Formation

Previous Serial Number: Same

Principal Investigator: Dr. Bruce Merchant

Other Investigators: Project Subtitle I: Dr. John Inman
Dr. Ramesh Srivastava
Project Subtitle II: Dr. John Inman
Dr. Donald Mosier
Project Subtitle III: Dr. Alfred Steinberg

Cooperating Units: Dr. Y. B. Kim, Dept. of Microbiology, University of Minnesota, Minneapolis, Minnesota
Dr. Latham Claflin, Dept. of Pathology, Washington University School of Medicine, Saint Louis, Missouri

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 1 7/12 | 4/12 | | 1 11/12 |
| Professional | 7/12* | 4/12 | | 11/12 |
| Other | 1 | | | 1 |

* A total of approximately 5/12 of Dr. Merchant's time has been devoted to service functions in the following three capacities: 1. EEO counselor for NIAID, 2. Secretariat for NIAID Task Force on Immunology and Disease, and 3. Special Assistant in Allergy and Immunology Branch, EP/NIAID.

Considerable additional scientific time has been spent in introducing nude (athymic) mice to the NIH. These mice were obtained from Dr. John Jutila of the University of Montana. After undergoing a 6 month quarantine at the NIH farm at Poolesville they were transferred to the regular "specific pathogen-free" production unit on the NIH reservation. Limited numbers of Nude x NIH albino mice are now in production. Breeding programs have been initiated which will "graft" the nude genotype into 8 standard strains of inbred mice. When such special nude x inbred strains become available they will comprise a major genetic resource for all NIH scientists. We are cooperating closely with Dr. Carl T. Hansen of the Veterinary Resources Branch of DRS in this special service project.

Project Subtitle I: Cellular Participation in the Immune Response to Chemically Defined Antigens

Project Description:

Objectives:

1. To determine whether the IgM antibody-forming cell response to defined chemical haptens is capable of specificity maturation.

2. To determine the magnitude and extent of non-specific factors operative in IgM antibody-forming cell activation.

Methods Employed:

Inbred mice are immunized with large structurally defined azo-haptens coupled to proteinacious carriers, e.g., para-azobenzenearsonate tyrosylglycylglycine coupled to duck erythrocyte stromata (A-TGG-DRC) or dinitrophenylalanylglycylglycine keyhole limpet hemocyanin (Dnp-AG-KLH). Mouse spleens are collected after intervals of four days to several months; spleen cell suspensions are prepared and hapten specific hemolytic plaque forming cell assays are performed to detect plaque forming cells reactive with the immunizing hapten or with any of a dozen structurally related, unrelated or structurally intermediate haptenic determinants. Comparable hapten-specific IgM profile determinations are carried out on appropriate control groups including a group receiving only saline injections. The numbers of hapten-specific plaque forming cells per million nucleated spleen cells are determined for each experimental and control group and a stimulation index (an experimental to control, E/C, ratio) is established for each haptenic reactivity. By plotting the experimental to control ratios for specific and non-specific IgM plaque forming cells over the time course of the experiment, the trend for specific, for semi-specific and for non-specific IgM plaque forming cell activation can be defined and documented. The large structurally defined aso-haptens employed in this work have been described in detail in previous annual reports. Suffice it to say that they have been selected so as to represent groups of haptens which either have much, little, or intermediate structural similarity. This allows designation of the assay haptens into three categories, viz, 1. haptens closely related to the immunizing hapten, 2. disparate or structurally unrelated haptens, and 3. haptens of intermediate structure. For any given experiment a minimum of three or a maximum of seven haptens are designated in each category, but normally either four or five haptens comprise each of the three diverse specificity categories. Definition of the mean stimulation index for groups of haptens allows evaluation of the magnitude and time course of quasi-specific, semi-specific, and non-specific IgM plaque forming cell activations which occur during the process of immunization.

Major Findings:

1. Specific, quasi-specific, semi-specific and non-specific IgM

plaque forming cell activations all occur during the initial days of IgM antibody formation.

2. Specific IgM plaque forming cell activation often comprises a minor component of total IgM activation at the usual four to five day assay interval following immunization.

3. Despite its poor start, specific IgM PFC activation gradually tends to become predominant over the next few days to weeks and specific IgM plaque forming cells usually disclose the most favorable E/C ratio after two to three weeks of immunization.

4. Favorable specific IgM plaque forming cell E/C ratios are potentially transient and may be lost after a rest period of about 3 months.

5. Quasi-specific, semi-specific and non-specific IgM PFC activation is most prominent during the first week after immunization. Such activation often peaks on the 4th day post-injection. Thereafter, semi-specific and non-specific E/C ratios decline precipitously; however, the quasi-specific IgM PFC stimulation index tends to parallel the specific PFC index but at a lower level.

6. Semi-specific and non-specific IgM plaque forming cell populations are relatively refractory to reactivation at two weeks following initial activation. These non-specific and semi-specific precursor cell populations have largely recovered from this refractory period by three months after initial immunization.

Significance to Biomedical Research and the Program of the Institute:

The present findings confirm and extend our previous observations which have documented a cross section of the repertoire of IgM specificities that both rabbits and mice marshall in response to chemically defined haptenic stimuli. They disclose that IgM PFC responses do clearly trend toward specificity. In addition, they document the existence of truly non-specific IgM PFC responses. However, they demonstrate that these seemingly adverse cellular commitments are transient in nature.

Such findings are relevant to the physiology of antibody forming cell precursor activation. They are consistent with the concept proposed by several investigators that both specific and non-specific activating factors are released by T cells as a result of their contact with antigen. Whether T cells are responsible for the presently identified transient non-specific activation of B cells is uncertain; however, future experiments in athymic mice should serve to answer this question.

Non-specific activation of IgM committed B cells could significantly perturb specific immunization with antigens which engender predominantly IgM responses. The period of refractoriness to non-specific stimulation is probably relevant to the design of both clinical and experimental immunization protocols for the use of such antigens.

Proposed Course of Project:

A cell transfer system will be utilized to evaluate the specific and non-specific activation components of the IgM response to chemically defined large azo-haptens. We plan to use both single and double microchambers inserted into the peritoneal cavities of both nude (athymic) and heterozygous mice in order to evaluate possible B and T cell contributions to specific and non-specific IgM activation.

Project Subtitle II: Genetic and Environmental Factors Contributing to Natural Hapten-specific Antibody Formation.

Project Description:

Objectives:

To identify possible genetic controls over natural IgM antibody formation, and to elucidate as many as possible of the environmental factors which influence natural IgM antibody production.

Methods Employed:

The basic experimental and assay methods are essentially similar to those outlined under subtitle I. The germfree animal production facility has been cooperative in supplying modest numbers of inbred and outbred germfree mice essential to these studies. The use of fetal mice requires several additional precautions and inclusion of several special control protocols.

Major Findings:

The hapten-specific plaque activity profile constitutes a particularly useful tool for the study of natural "spontaneous" IgM antibody production. Only about 1 in 100 murine tests normally discloses the presence of "spontaneous" natural IgG antibody production. Thus, these studies have been restricted to the study of naturally occurring hapten specific IgM antibody releasing cells. Our studies have shown that such naturally occurring IgM releasing plaque forming cells reactive with from most to all of the synthetic chemical azo-haptens employed in the test profile are present in mice, rats, guinea pigs, rabbits and humans. Previous work from this laboratory has demonstrated that the hapten specific IgM "background" profiles vary from individual mouse to mouse even if they are inbred and furthermore that such background profiles vary with time. These profiles are generally reflective of immune potential. They are low in the neonatal period, attain maxiumum proportions during mid to late adulthood and decline considerably during old age. Current studies have been carried out in conventional, germfree and fetal mice and will be reported under the corresponding headings.

a) Studies in conventional mice.

1. Background IgM hapten specific plaque profiles are affected by environment. Mice of the same age, sex and strain, maintained in different animal rooms disclose different commitments in their IgM hapten-specific background profiles.

2. These background profiles can be elevated significantly at 4 days by injections of endotoxin, Freund's incomplete adjuvant, or allogeneic cells. Bacterial lipopolysaccharides obtained from the same bacterial strain but by different extraction procedures may cause either elevation or reduction of background IgM plaque forming cell profiles.

3. Background profiles are refractory to the effects of 550 R irradiation for a period of up to eight days. Although such irradiation may cause a 50 fold reduction in numbers of nucleated cells within the spleen there is no coexisting reduction in the numbers of background IgM plaque forming cells.

4. Specific pathogen-free mice disclose background IgM profiles which are intermediate between those observed in germfree and those observed in conventional mice of the same age, sex and strain. Conventionalization of specific pathogen-free mice for periods of four days leads to marked fluctuations in their background profiles, most of which involve increases in given haptenic specificities.

5. Background hapten specific PFC activities are regularly elevated in nude (athymic) mice over those activities expressed by heterozygous, normal litter-mate controls.

b) Studies in germfree rodents.

1. The background IgM profiles of germfree mice and rats are extremely similar (almost identical) from one individual to another if they are of the same age, sex and strain. Such findings suggest a strong genetic control over the differentiation and activity of IgM background plaque forming cells.

2. Germfree mice and rats of outbred or random bred strains show highly variable IgM background profiles between individuals of the same age, sex and litter.

3. Germfree mice disclosing high background IgM activities to certain haptens, e.g., DNP or TNP-AGG were refractory to immunization with a corresponding hapten implying refractoriness to stimulation with DNP-AGG-KLH which was shown to be immunogenic in conventional mice of the same age, sex and strain. The reproducibility of this finding is currently being checked.

4. After conventionalization (i.e., 4 days of exposure to conventional mice of the same age, sex and strain.), previously germfree mice disclosed background profiles which are intermediate between their usual typical germfree and conventional background profiles.

5. Conventional or stock mice which have been exposed for four day intervals to previously germfree mice of the same age, sex and strain (i.e. conventional mice employed to conventionalize germfree mice) disclose extraordinary 5 to 10 fold increases in their background IgM plaque profiles. This result cannot readily be attributed to bacterial contamination of conventional mice by exposure to germfree mice, although it could conceivably be the result of lateral virus transmission from germfree to conventional mice. The reproducibility of this serendipitous observation is currently under study.

c) Studies in fetal mice.

1. Background plaque forming cells first appear (ostensibly spontaneously) at 19 days gestation of fetal mice. Initially, only 1 or 2 of the fifteen test specificities can be detected. By the 21st (final) day of gestation, 6 different specificities were detected in each of two inbred mouse strains. These profiles were profoundly different between the two strains but were highly reproducible within either strain.

2. Activity for any given specificity always appears predictably on the 19th, 20th or the 21st day of gestation. The specificity profiles of fetal mice bear no apparent relationship to coexisting hapten specificity profiles in the spleens of their gestating mothers.

3. Background IgM profiles in 19 to 21 day fetal mice are unaffected by active immunization of the gestating female. Active immunization of maternal mice never caused the precocious appearance of background hapten specific PFC in fetal mice of 14 to 18 days gestation.

4. Pools of fetal lymphoid cells from outbred mice also disclosed background hapten specific PFC on day 19; however by day 21 nine of the fifteen test specificities were normally demonstrable. Absolute plaque forming cell values in lymphoid cell pools from outbred fetal mice normally ranged lower than those observed in inbred fetuses.

Significance to Biomedical Research and the Program of the Institute:

Since both inbred and outbred germfree mice share the same environment and consume the same diet, the finding of identical IgM background profiles in inbred and highly variable profiles in outbred mice indicates a strong genetic control over expression of background IgM plaque forming cell commitments. Nonetheless, studies with conventionalized germfree mice and with conventional mice of the same age, sex and strain disclose numerous environmental factors which can profoundly affect the expression of background IgM natural hapten specific antibody. Thus, it appears that these unique B cells are under definite genetic regulation; however, like other B cells their precursors appear highly receptive to environmental stimuli.

The findings in fetal mice strongly suggest that genetic control over the differentiative development of IgM committed precursor cells are meant to be exerted prior to birth, and that this schedule of differentiative development is unique to each inbred mouse strain. Such observations lend support to germline theories of antibody formation; however, such support cannot be considered conclusive. Vertically transmitted viruses conceivably could cause similar manifestations. Since many vertically transmitted viruses may yet remain undiscovered, final clear resolution of this question becomes difficult.

The finding that background IgM hapten specific activities are regularly elevated in nude mice suggests that they may be performing a compensatory function in these mice which are immunologic cripples as a result of their total T cell deficit.

Since IgM antibody production is evolutionally, ontogenetically and very often circumstantially, the first immunoglobulin class produced in active immune responses, a complete understanding of the genetic and environmental factors which influence it is most important. The present project probably constitutes the most comprehensive study of natural IgM antibody formation in contemporary immunology. As such, it should comprise a useful point of reference for studies from leading laboratories which seek to identify precise factors influencing B cell activation.

Proposed Course of Project:

Studies of germfree and specific pathogen free mice will be continued. Attempts will be made to manipulate background IgM hapten specific PFC numbers in germfree mice along the lines of the manipulations already accomplished in conventional mice. Isolation and exposure experiments will be carried out in order to ascertain whether elevated background PFC activities in germfree mice may be referable to the presence of viral agents. Fetal cells will be studied in a transfer system involving microchambers inserted into the peritoneal cavities of both syngeneic and allogeneic recipients. If the expression of IgM PFC by fetal cells can be accomplished with the microchamber system, it will be useful to ascertain whether characterisitic profiles arise only in the environment of the syngeneic host or whether such unique profiles also will be expressed inside microchambers contained in an allogeneic environment.

Project Subtitle III: Dynamics of Natural IgM Antibody Production in Animals and Patients with Autoimmune Disease.

Project Description:

Objectives:

1. To determine whether patients with lupus erythematosus demonstrate increased natural IgM antibody commitments to specific haptens as is the case with NZB and ZBW female mice.

2. To determine whether the unusually high background PFC commitments present in NZB and ZBW female mice can be manipulated experimentally.

Methods Employed:

The methods depend on the hemolytic PFC assay performed with the large chemically defined azohaptens as described above. Hemagglutination assays employing the large azohapten conjugated SRC are also used. Previously reported experimental observations in our laboratory had demonstrated that adult NZB and (NZBxNZW)$F_1$ [B/W] female mice disclosed unusually high IgM PFC commitments to many or most of the azohapten determinants employed in our screening profile. Only nude mice normally disclosed hapten specific background PFC commitments of comparable magnitude. Nude mice are known to suffer a complete T cell deficit. Experiments from Dr. Steinberg's laboratory indicate that NZB and NZBW female mice also develop a T cell deficit which is progressive with advancing age. Dr. Steinberg has previously shown that transfer of syngeneic young thymus cells into old mice of these two New Zealand strains leads to amelioration of their autoimmune disease. Similar experiments have been conducted in order to ascertain the effect of transferred syngeneic cells on the expression of background IgM hapten specific PFC activities. Other studies are underway using circulating leukocytes from human patients.

Major Findings:

Since as reported last year the standard murine models of humoral autoimmune disease demonstrated consistently elevated background hapten specific IgM activities, it seemed reasonable to inquire whether the same might be true in the prototype human disease, lupus erythematosus. Acquisition of splenic material from human patients is of course impractical; hence we undertook to study human leukocytes from circulating peripheral blood. Twenty patients thus far have been studied in collaboration with Dr. Fred Steinberg, and the major findings are summarized below.

1. Hapten specific background plaque forming cells are regularly detectable in the circulating peripheral leukocyte compartment.

2. Marked variations in background hapten specific plaque activity exist from patient to patient.

3. Some normal patients, especially females, show high background plaque forming cell activities but the very highest activities thus far were seen in lupus patients.

4. No clear correlation of plaque forming cell activity and severity of disease is yet possible.

5. Lupus patients frequently demonstrated elevated activities for three haptens, benzoyltyrosylglycylglysine, carbobenzoxyalanylglycylglysine, and the unsubstituted acetyltyrosylglycylglysine.

In BW female mice, transfer of 50 x $10^6$ syngeneic young thymus cells led to from 2 to 12 fold elevations in specific background plaque forming cell activities at four days. These activities had subsided to 4 fold or less elevations by seven days after transfer. Only modest elevations were observed in comparable experiments with NZB mice.

This manipulative portion of the project is just getting under way. Many more cell transfer studies are contemplated. Efforts will be made to ascertain whether IgM hapten specific background profiles are refractory to reactivation by a second thymus cell transfer as is the case for nonspecific activation following immunization. Efforts will also be undertaken to discern positive or negative correlations between background hapten specific IgM profiles and the anti--nuclear or anti-nucleotide antibodies which coexist in these murine models of autoimmune disease.

Significance to Biomedical Research and the Program of the Institute:

Since background hapten specific plaque activites are regularly and profoundly elevated in NZB and BW female mice, the hypothesis was advanced that the basic pathologic lesion in these autoimmune mice might be operationally described as a broad spectrum proclivity to B cell activation. B cells susceptible to triggering by only the vaguest stimuli might ultimately be expected to express both anti-nuclear antibody and antibodies reactive with a wide range of synthetic chemical hapten determinants. It is still too early to ascertain whether this hypothesis will be supported or rejected by the experimental findings. However, additional experiments scheduled and in progress should permit such a determination.

Since the hapten specific IgM profile constitutes a precise and effective tool for evaluating the IgM status of an animal or individual, it should substantially improve our ability to detect short term variations and fluctuations in the IgM status of normal and autoimmune animals and pateints. Experiments with the mouse autoimmune model justify this opinion. However, further studies will be required in humans to ascertain how useful the hapten specific IgM profile will be in the investigation of humoral-mediated human autoimmune disease.

Proposed Course of Project:

Many further cellular and serologic studies will be undertaken with human material. It will be of primary importance to determine the normal range of background IgM profiles in normal patients. Further efforts will also be carried out to identify specific hapten reactivities which may correlate with exacerbation or amelioration of autoimmune disease processes. In the mouse model many more cell transfer studies are contemplated. The dynamics of IgM hapten specific profile activities will be ascertained following T cell transfer and following administration of anti-T cell antibody. Spleen cells from New Zealand mice contained in micro-diffusion chambers will also be transferred into syngeneic and allogeneic hosts in order to determine the influence of these environments upon expression of B cell activity as manifested by the hapten specific IgM profiles.

Publications:

Claflin, L. and Merchant, B.: Restricted maturation of antibody binding characteristics for hapten-specific IgM plaque-forming cells in mice. Cell. Immunol. 5: 209-220, 1972.

Claflin, L., Merchant, B. and Inman, J.: Antibody binding characteristics at the cellular level. I. Comparative maturation of hapten specific IgM and IgG plaque-forming cell populations. J. Immunol. 110: 241-251, 1973.

Claflin, L. and Merchant, B.: Antibody binding characteristics at the cellular level. II. Presence of cells secreting high affinity IgG antibody at the onset of the immune response. J. Immunol. 110: 252-261, 1973.

Merchant, B., Inman, J.K. and Claflin, L.: Evidence for genetic control of spontaneous hapten specific IgM antibody production. In McDevitt, H. and Landy, M. (Eds). Genetic Control of Immune Responsiveness, New York, N.Y. Academic Press, 1972, pp 195-201.

Serial No. NIAID - 145
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on Immunological Specificity

Previous Serial No.: Same

Principal Investigator: Dr. John K. Inman

Other Investigators: Project Subtitle I: Dr. Bruce Merchant
Dr. Latham Claflin
Dr. Ramesh C. Srivastava
Project Subtitle II: Dr. Ettore Appella (NCI)
Dr. Ramesh Srivastava
Dr. David Alling

Cooperating Units: Dr. Ettore Appella, Laboratory of Cell Biology, National Cancer Institute

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 2 1/4 | | | 2 1/4 |
| Professional | 1 1/4 | | | 1 1/4 |
| Other | 1 | | | 1 |

Project Subtitle I: Cellular Studies on the Development of Specificity in Humoral Immune Responses

Project Description:

Objectives:

To apply and extend newer methods in hapten-specific hemolytic plaque assays to studies of antibody secreting cells. To direct these studies toward questions concerning: 1) the specificity of precursor cell activation, 2) the nature of immunological specificity in interactions at cell surfaces, 3) antibody specificity, affinity and avidity during the course of immune responses, and 4) ontogenetic development of specific immunological competence.

Methods Employed:

A group of specially synthesized, large, haptenic compounds were employed to specifically sensitize sheep erythrocytes used in hemolytic plaque assays. Immunizing antigens were also prepared with the same haptens. Complete details for carrying out these methods have been published recently (see Publications: Inman, et al., Immunochem. - 2 papers; Rouques, et al., Int. Arch. Allergy; cf also my last annual report).

Major Findings:

Major findings will be discussed in the annual report of Dr. Merchant. This work has been carried out entirely in collaboration with him. An additional year of experience with the synthesis and coupling of large haptens to cells and proteins has amply demonstrated the effectiveness and reproducibility of the methods that were developed earlier in the study.

Significance to Biomedical Research and the Program of the Institute:

The study has provided some new and generally applicable methods for preparing specific immunological reagents which can be used in most areas of basic immunological research. They may also find use in applied research concerned with manipulation of immune responses. Application of our methods to hemolytic plaque assays have led to a number of new insights into the nature and development of specificity in humoral immune responses.

Proposed Course of Project:

Our current studies on the maturation of specificity during immune responses will be continued. Additional large haptens will be prepared and studied, and some specially designed antigens will be synthesized. For further details see the corresponding section of Dr. Merchant's report.

Project Subtitle II: The Specificity of Antibodies: Structural and Genetic Studies

Project Description:

Methods Employed:

Preliminary evaluation of the probability model for cross reactions was carried out with the aid of our Wang calculator using certain approximation formulas for large factorials. Dr. Alling and I transformed the functions into non-factorial expressions which were programmed for the Control Data Corp. 3100 digital computer. Rapid progress was made thereafter in testing the basic concepts in terms of realistic values for the various parameters.

Adsorbents for sensitive binding (screening) assays were designed. The primary adsorbent was based on a cross-linked polyacrylamide matrix. A

new compound (6-bromoacetamidocaproic acid) was synthesized to serve as an intermediate for introducing a spaced-out, primary functional group for attaching the screening haptens. The binding assay will consist of interacting separate gel-hapten conjugates with $^{125}I$-labeled, specifically purified antibodies. Unbound antibody will be washed out and the gel phase counted. A new iodination procedure for the antibody labeling is being investigated. The work involves synthesis of reactive compounds containing iodine and kinetic studies on exchange of iodine into and out of these compounds.

The studies with a new approach to identification and quantitation of peptide sequential (Edman) degradation products has employed many synthetic steps commonly used in peptide chemistry. Identification of compounds has been carried out so far on thin layer chromatographic plates. Degradation products from both manual and automatic operations have been tested.

Major Findings:

A new theoretical treatment of antibody and serological specificity was developed from the concept of multispecificity for individual antibody combining sites as put forward by Talmage (in 1959), Glazer (in 1970) and Rosenstein, et al. (in 1972). By applying principles of combinatorial analysis I was able to derive a general expression for the probabilities of serological cross reactions between structurally unrelated antigenic determinants. With the help of Dr. David Alling an extensive computer evaluation of the probability functions was carried out. With the results it was possible to show that an individual animal, with a repertoire limited to $10^4$ to $10^6$ structurally distinct antibody combining sites, could respond with a specific population of antibodies to any one of an essentially unlimited number of antigens. If in fact, nature has taken this conservative course in providing for serological specificity, then it will be somewhat easier to account for the genetic basis of antibody diversity than has been previously supposed. Thus, the burden may be lifted of having to explain with germline or somatic-hypermutation mechanisms how many millions of different antibody structures would be made.

The quantitative theory also predicts that sensitive binding assays will find rather frequent, very minor cross reactions between specific, heterogeneous populations of antibodies and various structurally unrelated test haptens. If such results are obtained, the concept of multispecificity will be verified. Measurements of the frequency of such cross reactions will help to set both upper and lower limits to the number of antibodies (as combining site structures) that an animal can make.

Work is underway to set up a screening program for disparate structure cross reactions. Since the assay procedure will employ radioiodinated antibodies some thought has been given to the method of attachment of the label. Existing methods for labeling proteins involve ring iodination of tyrosine residues. Reactive tyrosine residues have often been associated with antibody combining sites and therefore should not be altered in studies such as these. I have considered the possibility of labeling at less sensitive loci. A

method was designed and worked out whereby $^{125}I^-$ can be rapidly exchanged into a reactive iodoalkanoyl compound and later used to form an amide linkage with a free amino group on the antibody molecule. The method was tested and found to be satisfactory when using the compound, N-(6-iodocaproyl) succinimide. Another important advantage of the method is the extreme gentleness of the labeling conditions such as use of aqueous, neutral buffers without any oxidizing reagent or oxidative enzyme system. It is expected that this approach may be especially suitable for extrinsic labeling of membrane protein on viable cells. Work is currently centered on finding the optimal structure for the alkanoyl radical and the activating group (leaving group for effecting the labeling reaction with protein).

A longer range goal of this project has been to obtain amino acid sequence data of variable regions of restricted or homogeneous antibody preparations. To this end I have been interested in opportunities to improve existing methodology in the primary structure determination of proteins. One of the critical needs at present is for a single, satisfactory method to identify and quantitate amino acid derivatives as they emerge from automated sequential degradations by the widely used method of Edman and Begg. The presenting derivatives are very unstable thiazolinones which must be first converted with aqueous acids to more stable compounds. The procedures used result in considerable losses of some common amino acids. During the past year I found that thiazolinones can be rapidly converted under very mild conditions to stable amide derivatives by a variety of nucleophilic reagents. One of these reagents has shown considerable promise and is now under extensive investigation in collaboration with Dr. Ettore Appella (NCI). We are testing high-pressure liquid chromatographic systems for separating and quantitating the new derivatives. The latter can be formed in programmed operations of the Beckman automated sequencer.

Significance to Biomedical Research and the Program of the Institute:

The work with disparate structure cross reactions and the primary structure of antibodies is aimed at clarifying or correcting some fundamental concepts concerning 1) the specificity of antibodies and 2) the origin of diversity of antibody structure. These concepts underpin nearly every aspect of basic immunological research. I believe that many views commonly held in regard to the above concepts are misleading and incorrect. It should be a matter of utmost priority to develop and apply new methods of biochemical and immunochemical research toward elucidating and resolving fundamental questions remaining in the area of specificity of immune reactions.

Proposed Course of Project:

The radioiodination study will be completed with the assistance of Dr. Srivastava in the next few months. Various materials and procedural details of screening assay will be tested using labeled rabbit anti-azobenzene-arsonate antibodies. Hapten-polyacrylamide conjugates, to serve as a screening panel for cross reactions, will be prepared with the assistance of Miss Duntley.

As these become available actual screening tests will be performed. Rabbits are now under immunization for this purpose.

Disparate structure cross reactions (DCR) will be used in experiments to cross immunize animals with two or more unrelated haptens in order to raise quantities of monoclonal antibody for primary structure studies. The feasibility of carrying out this interesting manipulation of a specific humoral response has been indicated through results of very recent experiments at Yale by F.F. Richards et al. and at Washington University by Latham Claflin.

After methods have been tested in rabbits, studies on DCR will be continued in mice; at this juncture, DCR's will be employed as genetic markers in much the same way that anti-idiotypic antisera are being used to study inheritance patterns of variable region structural genes.

Work will be continued on the development of the new identification-quantitation method for Edman degradations. Emphasis will be placed on use of liquid chromatographic methods using recently developed adsorbents and equipment which are commercially available. Dr. Appella now has the DuPont Model 830 Liquid Chromatograph with gradient attachment and a highly sensitive UV detection system which will be ideal for further development of the method.

Publications:

Rouques, R., Inman, J.K. and Merchant, B.: Detection of cells secreting antibodies more reactive with an alternate structure than with the immunizing hapten. Int. Arch. Allergy & Appl. Immunol. 42: 852-870,

Inman, J.K.: Specific adsorption and affinity chromatography of cells. In Cohen, S. and McCluskey, R.T. (Eds.): Mechanisms of Cellular Immunity. New York, N.Y., Wiley-Interscience, John Wiley & Sons, Inc., In Press, 1973.

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Serial No. NIAID - 146
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1973

Project Title: Chemical, Genetic, and Immunochemical Studies of Immunoglobulins: Use of Genetic Markers in Studies of Lymphoid Differentiation and the Regulation of Gene Expression and Immune Responses

Previous Serial Number: Same

Principal Investigators: Rose G. Mage, Ph.D. and Glendowlyn O. Young-Cooper, M.S.

Other Investigators:
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Dr. M. Harrison LI-NIAID
Dr. J. K. Inman LI-NIAID
Dr. M. Waxdal LI-NIAID
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Cooperating Units:
Dr. S. Dray, University of Illinois Medical Center Chicago, Illinois
Dr. M. Freedman, University of Toronto, Toronto, Canada
Dr. S. Tosi, Laboratorio di Biologia Cellulare del C.N.R., Rome, Italy
Dr. A. Chersi, Istituto Regina Elena, Rome, Italy
Dr. G. Siskind, Cornell University Medical College, New York, N.Y.

In addition, this year the following investigators have used rabbits of defined allotype which we have supplied and/or have received specific reagents which we have prepared: Dr. R. Cathou, Boston, Mass., Dr. J. Cebra, Baltimore, Md., Dr. J.W. Chiao, New York, N.Y., Dr. M. Feldman, London, England, Dr. A. Flaharty, Pomona, Calif., Dr. M. Goldman, Boston, Mass., Dr. R. Goodfleisch, Madison, Wisc., Dr. I. Green, NIAID, Dr. M. Johnson, NIDR, Dr. M. Koshland, Berkeley, Calif., Dr. A. Lawton, Birmingham, Ala., Dr. P. Liberti, Phila., Pa., Dr. W. Mandy, Austin, Tex., Dr. H. Metzger, NIAMD, Dr. J. Oppenheim, NIDR, Dr. W. Paul, NIAID, Dr. S. Pestka, Nutley, N.J., Dr. J. Robbins, NICHD, Dr. D. Sachs, NCI, Dr. N. Talal, San Francisco, Calif., Dr. E. Unanue, Boston, Mass., Dr. H. Wortis, Boston, Mass., Dr. B. Wolf, Phila, Pa. and Dr. J. Young, Richmond, Calif. We have also typed samples for numerous investigators.

| Man Years: | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 5 | | 1/2 | 5 1/2 |
| Professional | 4 | | 1/2 | 4 1/2 |
| Other | 1 | | | 1 |

Project Description:

Objectives:

To define by combined immunological and chemical approaches, the chemical nature of structural differences between genetically controlled polymorphic forms of immunoglobulins and between different classes, subclasses, types and subtypes of their polypeptide chains. To utilize this information about the location and chemical nature of the markers on various chains in performing and interpreting breeding studies. To utilize defined reagents for various chains and markers in affecting phenotypic expression of alleles or chain types. The overall objective is to understand the genetic mechanisms which lead to the synthesis of highly specific antibody molecules and the nature of the genes which influence and control immune responses.

Methods Employed:

Rabbits of known allotype are bred: to produce homozygous rabbits of all combinations at four or more genetic loci; to produce heterozygotes for use in specific experiments and as F1 progeny in linkage studies; to produce F2 and backcross progeny in linkage studies; to produce allotype-suppressed rabbits; and to study the genetics of the immune responses to pneumococcal vaccines, DNP-protein conjugates, and other antigens.

Sera of rabbits from the above categories are collected and typed by gel diffusion and hemagglutination techniques for allotypes. The sera are used as sources of IgG and light and heavy polypeptide chains for chemical sequence studies and for immunizations of rabbits and other animals.

Rabbits of defined allotype are immunized with IgG, light chains or other antigens such as lysozyme, pneumococcal vaccines, hapten-protein conjugates and (for idiotype studies) purified antibodies or IgG fractions and subfractions. Antisera are tested by qualitative and quantitative immunologic and electrophoretic techniques.

Allotypes, types, subtypes and idiotypes are quantitated by: radial immunodiffusion; hemagglutination inhibition; quantitative precipitation of iodine labeled proteins with specific antisera; binding of labeled proteins

to immunoabsorbents; inhibition of binding of the labeled antigens to immunoabsorbents. Immunoabsorbents are also prepared and used to purify antibodies, antigens and peptides of known specificity.

Cells from newborn and developing normal and allotype suppressed homozygous and heterozygous rabbits are labeled with iodinated or fluorescein-conjugated anti-allotype reagents. They are also cultured in vitro and assayed for incorporation of labeled amino acids or tritiated thymidine after exposure to a variety of materials including mitogens and anti-allotype reagents.

Purified light and heavy chains or fragments of these are obtained from IgG of normal sera or specifically purified antibodies of known allotype as well as from sera of allotype-suppressed rabbits. These are studied using a variety of methods for sequence determination including automated sequencing.

In order to carry out all the studies, we breed and maintain large numbers of rabbits in our own colonies. We produce and characterize large volumes of antisera including anti-allotype antisera made with a variety of donor and recipient combinations, anti-idiotype antisera, antibodies of restricted heterogeneity, and other anti-immunoglobulin reagents. We purify normal and antibody immunoglobulins from large volumes of rabbit sera for use as immunizing antigens, for structural studies, and for other studies described below.

Major Findings:

Rabbits from different breeding groups and with different genetic back- and allotypes were selected for their ability to produce structurally restricted anti-polysaccharide antibodies when immunized with either type III or type VIII pneumococcal vaccines. A significantly greater proportion of animals exhibiting this trait were found among the close relative and offspring of restricted responders compared to randomly chosen animals. The degree of antibody restriction was not correlated with the magnitude of the immune response. The trait was observed in animals of both sexes and there was no obligate association with any a or b locus allotype. Some rabbits immunized with type III pneumococcal vaccine produce restricted components that did not have apparent specificity for type III polysaccharide or pneumococcal C substance. Isoelectric spectra of antibodies produced by individual rabbits were compared on thin layer acrylamide slabs. No two patterns were identical when different rabbits were compared although some siblings did appear to produce similar components. Several rabbits which were restimulated after 1 to 2 years produced antibodies with isoelectric spectra identical to those originally produced.

The immunoglobulins from selected heterozygous and homozygous rabbits hyperimmunized with type III or type VIII pneumococcal vaccines were fractionated by preparative liquid isoelectric focusing (IEF) at various pH ranges. From each antiserum, a number of focused fractions with different pI

values were obtained and many of the major fractions had reduced electrophoretic, structural, allotypic and idiotypic heterogeneity compared to the unfractionated immunoglobulins. We have demonstrated that this method provides a means of preparatively obtaining different antibody fractions from a single antiserum.

Light and heavy polypeptide chains have been prepared from isolated immunoglobulin fractions of sera with antibodies of restricted heterogeneity. The amino terminal sequence of several light chains has been determined, using a Beckman sequencer (with Dr. E. Appella). In addition a full sequence determination of a b4 light chain is being attempted (S. Carta and E. Appella). The heavy chains of several of the antibodies are being used in studies of the chemical basis of a locus allotypic determinants (with E. Appella and M. Carta-Sorcini). The heavy chains from both normal homozygous rabbits and from homozygous rabbits producing anti-SIII or anti-SVIII antibodies of restricted heterogeneity were trypsin digested after partial citraconylation to block lysine residues. The mixture of tryptic peptides specifically inhibited the appropriate a-anti-a precipitin reaction in Ouchterlony gel diffusion. The peptides were separated on G-75 Sephadex, and one or more fractions were obtained with specific inhibitory activity. Chemical characterization of active fractions from normal IgG was complicated by the heterogeneity of sequence of the region from which the peptides appear to be derived. Antibodies of restricted heterogeneity are providing better starting material for the isolation of active peptides. Even from this source, some heterogeneity is still present and chemical isolation techniques did not provide material pure enough for sequence determination. We therefore turned to immunochemical purification. We isolated anti-a3 antibody by affinity chromatography on a3 IgG-Sepharose. This antibody was then coupled to Sepharose and the mixture of tryptic peptides from a3 heavy chain was passed through the affinity column. A peptide fraction which bound to the immunoabsorbent was eluted with acetic acid (0.1 N) and found to have specific inhibitory activity. A peptide with the same amino acid composition was also purified from a G-75 Sephadex column fraction with inhibitory activity. The chemical characterization and localization of the peptide in the heavy chain sequence is now in progress. The peptide is 25-30 residues long, contains cystine, and has about twice as much arginine as lysine at the carboxyl terminal. Classical cyanogen bromide cleavage products of the same heavy chain from which a3 active peptide has been isolated have already been prepared, and the determination of their sequence is in progress. We therefore hope to be able to unequivocally place the a3 active peptide in the heavy chain sequence. Quantitative inhibition of binding assays have been developed which permit comparisons of the inhibitory activity of peptides. The binding of iodine labeled heavy chain to anti-allotype immunoabsorbent is inhibited by the unlabeled heavy chain, IgG, and by peptides derived from the heavy chain.

Since at least two recombinations between $V_H$ (a-locus allotypes) and $C_\gamma$ (de, 11,12,14,15) have been observed in laboratory breeding, it is possible

that the distances between genes for variable regions and constant regions of rabbit heavy chains are comparable to those separating the D and K portions of the H-2 gene complex or the analogous LA and Four segregant series of the human HL-A region. We are continuing to test for recombination in breeding experiments. The recombination frequency between $V_H$ and $C_\gamma$ in rabbits based on only two observed recombinants is imprecise. Two recombinants among 628 observed informative offspring = 0.3% (0.04-1.2%, 95% confidence limits) which compares with 0.4% for H-2 D and K and 0.8% for LA and Four. It may well be that the Ig heavy chain genetic region is comparable in size to the genetic regions encompassing serologically detectable transplantation antigens, MLC lymphocyte antigens, Ir genes etc.. Initial chemical characterizations of the IgG produced by normal 1,14/1,14 progeny derived from the sire of a recombinant rabbit and 2,14/2,14 progeny representing a recombinant type of $\gamma$ chain were completed. The A14 and A15 allotypes correlate with a threonine-alanine interchange at position 309 in the $\gamma$ chain. We confirmed that the "recombinant" IgG with serologically detectable A14 did indeed have threonine at position 309. To compare a2 IgG with the original a1, we determined the amino acid compositions of the Fd fragments obtained from the two types. The chemical compositions of the a1 and a2 Fd fragments supported the serological assignment of a2 to the recombinant $\gamma$ chain. The molecular weights of the heavy chains from the IgG of the normal and recombinant progeny were indistinguishable by a method which could have detected a difference of 2500 daltons. The colostral IgA produced by the original recombinant female was also studied since typing for a allotypes was originally done with serum only in order to see whether any of the original a1 allotype might still be expressed on IgA. The IgA clearly carried the a2 allotype from the sire and a3 from the dam. None of the original a1 allotype of the sire was detectable with any of six different anti-a1 antisera that were used in the tests.

In order to examine the precursor-product relationship between membrane associated immunoglobulin and secreted immunoglobulin of a given allotype, and to explore the cellular mechanisms involved in allotype suppression, we have studied the ontogeny of lymphocyte membrane-associated immunoglobulin and circulating immunoglobulin of the same allotype in normal $\underline{b^4b^5}$ neonatal rabbits and in allotype suppressed rabbits, as well as the effect of thymectomy on allotype suppression. In normal $\underline{b^4b^5}$ rabbits, cells bearing endogenously synthesized Ig of paternal allotype were present in peripheral blood (6-12% fluorescent positive cells), spleen (11-12%), and appendix (~ 5%) shortly after birth. Thus a potential target cell for suppressing anti-allotype antibodies, the cell bearing Ig of paternal allotype, is present during the crucial perinatal period when anti-allotype antibodies can initiate chronic allotype suppression. Within 24 hrs. of injection of b9 anti-b5 into newborn $\underline{b^4b^5}$ rabbits, cells bearing paternal b5 Ig were no longer demonstrable in peripheral blood, spleen or bone marrow. Cells bearing maternal b4 Ig were not affected. The suppressing b9 anti-b5 antibodies were not detectable on the surface of cells in these organs by sensitive fluorescent staining with b4b5 anti-b9 demonstrating that the injected antibodies were not simply masking b5 determinants on the membranes of the target cells. The cells were either eliminated, or their membrane Ig was modulated from the surface and not replaced with the original light chain

form. Studies of suppressed $b^4b^5$ heterozygotes confirmed and extended previous studies of peripheral blood lymphocytes of suppressed homozygous rabbits. In both situations the appearance of secreted allotype of the suppressed type is chronologically linked to the prior appearance of allotype-bearing lymphocytes. Although during postnatal ontogeny, cells bearing endogenously synthesized Ig of paternal allotype are present from birth, circulating b5 Ig is not detectable until about 2 weeks of age. Similarly during escape from total allotype suppression, the appearance of b5 bearing lymphocytes in bone marrow and peripheral blood heralds the appearance of circulating b5 Ig. However in suppressed b5 homozygotes recovering from total allotype suppression the presence of adequate numbers of b5 bearing lymphocytes does not insure the production of commensurate amounts of circulating b5 Ig. By enzymatically stripping the membrane of Ig determinants and allowing regeneration of membrane-associated b5 in vitro, we demonstrated that most, if not all, of the b5 bearing cells in such recovering rabbits actively synthesize the b5 Ig molecules found on their membranes. Thus there is a true discrepancy between the proportion of lymphocytes with actively synthesized b5 Ig on their membrane and the proportion of b5 Ig in the serum. The explanation for this discrepancy is currently under investigation.

Neonatal thymectomy did not interfere with the establishment of chronic allotype suppression in b4b5 heterozygous rabbits. However, the thymectomized suppressed rabbits did not compensate with production of amounts of the maternal allotype comparable to those produced by non-thymectomized suppressed rabbits. We will pursue further the explanation for the requirement of thymus cells for production of normal compensatory amounts of the alternative allele.

Significance to Biomedical Research and the Program of the Institute:

These studies help to define the genetic mechanisms which lead to the synthesis of highly specific antibody molecules and the nature of the genes which influence and control immune responses. Allotypic markers are providing valuable reagents for the study of cell surface receptors and the role they play in the differentiation of antibody producing cells. These investigations help delineate the chemical basis for specificity of protein antigens and antibodies, the nature of structural genes for immunoglobulin polypeptide chains and the complexities of mammalian genetic systems. The breeding studies and the cellular studies which can be done with rabbits provide information applicable to the homologous human immunoglobulin system. In addition, rabbit allotypes are analogous in many respects to cellular isoantigens involved in transplantation, cell-cell recognition systems, tumor antigens and other cell surface markers. An understanding of the chemical nature of these antigenic types, the factors which control their genetic expression, and their biological roles is thus of broad fundamental interest.

Proposed Course of the Project:

We are continuing most of the studies described. In addition to continuation of present breeding and immunization groups, we are initiating production of several new anti-allotype and anti-idiotype reagents. We are

breeding rabbits which produce anti-PnIII and -PnVIII of restricted heterogeneity and continuing matings of rabbit groups which differ in the affinity of the anti-DNP antibodies they produce. We hope to chemicall characterize peptides with a-locus allotypic determinants from antibodies of restricted heterogeneity of each a allotype using methods which succeeded for the a3 peptide in order to compare their structures and localization. Since anti-idiotype antibodies are also being raised against the antibodies of restricted heterogeneity, we may also be able to isolate peptides with idiotypic determinants by methods similar to those used for the a allotypes. The purified anti-a allotype reagents we are preparing for the chemical studies will also be used for cellular studies. One collaborative study now being initiated (with Drs. E. Unanue, and M. Karnovsky, Harvard University) will search for $V_H$ (a-allotypes) determinants on rabbit T cells by sensitive antibody labeling techniques including freeze-etch electron microscopy. The reagents will also be used in continued studies of the mechanism of allotype suppression. Cell transfer studies to determine whether active suppressor cells mediate chronic allotype suppression have been hampered until now by the lack of histocompatible inbred strains as donors and recipients. We are now in possession of such strains (from Jackson Laboratory, Bar Harbor, Me.) and are breeding them and initiating such suppression studies.

Honors and Awards:

Member of the Editorial Boards of Immunochemistry and Federation Proceedings. Member of Allergy and Immunology Study Section. Invited participant, Symposium on Immunogenetics at Conference on the Plasma Proteins and Cellular Elements of the Blood, Center for Blood Research, and Colloquium on the Genetics of the Immunoglobulins and of the Immune Response, Institut Pasteur.

Publications:

Goldman, M.B. and Mage, R.G.: Purification and effectiveness of anti-allotype antibodies in suppression of rabbit immunoglobulin allotypes. Immunochem. 9: 513-524, 1972.

Winfield, J.B., Pincus, J.H. and Mage, R.G.: Persistence and characterization of idiotypes in pedigreed rabbits producing antibodies of restricted heterogeneity to pneumococcal polysaccharides. J. Immunol. 108 1278-1287, 1972.

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Werblin, T.P., Kim, Y.T., Mage, R., Benacerraf, B. and Siskind, G.W.: The generation of antibody diversity. II. Genetic influence on the binding of anti-hapten antibody. Immunol. In Press, 1973.

Elfenbein, G.J., Harrison, M.R. and Mage, R.G.: Demonstration of proliferation by guinea pig and rabbit bone marrow derived lymphocytes in vitro in response to stimulation by anti-immunoglobulin antisera. J. Immunol. In Press, 1973.

Mage, R.: Rabbit allotypes and the genetics of variable and constant regions of immunoglobulin heavy chains. Vox Sanguinis. In press, 1973.

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Mage, R.G.: Altered quantitative expression of immunoglobulin allotypes in rabbits. In: Jerne, N. Ed., Current Topics in Microbiology and Immunology Springer-Verlag, Heidelberg, in press, 1973.

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Serial No. NIAID - 147
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immunogenetics of Mouse Immunoglobulin

Previous Serial No.: Same

Principal Investigator: Miss Rose Lieberman

Other Investigators: Project Subtitle I: Dr. M. Potter, LCBGY, NCI
Mr. W. Humphrey, Jr., LI, NIAID
Project Subtitle II: Dr. M. Potter, LCBGY, NCI
Mr. W. Humphrey, Jr., LI, NIAID
Project Subtitle III:

Cooperating Units: Dr. J. Latham Claflin, Washington University, St. Louis, Missouri
Dr. J. M. Davie, Washington University, St. Louis, Missouri

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 1 | | | 1 |
| Professional | 1/2 | | | 1/2 |
| Other | 1/2 | | | 1/2 |

Project Subtitle I: Study of Immunoglobulin determinants in Mice with Homologous Antisera to Determine the Genetic Control of the structure of Immunoglobulin Molecules

Project Description:

Objectives:

1. To prepare homologous anti-allotypic and idiotypic (myeloma-specific) antisera highly specific for determinants on various parts of the different chains of mouse immunoglobulin molecules.

2. To study genetic control of immunoglobulin determinants.

3. To use genetically controlled immunoglobulin markers in studies of antibody synthesis.

4. To determine if identical molecules, based on biologic activity and identification of Fab determinants, can be found in myeloma proteins from independently induced plasma cell tumors.

Methods Employed:

1. Preparation of immunoglobulin immunogens (Fc, Fab and light chains) from normal and plasma cell tumor bearing inbred mice.

2. Immunization of inbred strains of mice with specific normal or myeloma immunoglobulin molecules or fragments of immunoglobulin molecules (chains).

3. Identification of immunoglobulin determinants with homologous antisera using immunoelectrophoresis, Oudin and Ouchterlony gel diffusion methods, radioisotope tagging, precipitin inhibition method, hemagglutination inhibition techniques and immunoabsorbents.

4. Selective breeding of mice to determine linkage, allelism, independent assortment and crossover of genes. Production of coisogenic strains to propagate and isolate genetic markers in a known genetic environment. Chromosome mapping of genetic markers of immunoglobulin determinants with other genetic markers as coat color, etc., to facilitate ease identification of immunoglobulin genetic markers and also to determine the location on the chromosomes of genes controlling specific immunoglobulin determinants.

5. Development of techniques employing immunoglobulin determinants as genetic markers in problems of transplantation, histocompatibility, transfusion reactions, immunization and antibody synthesis.

Major Findings:

Immunoglobulin determinants have been identified on the heavy chains of immunoglobulin molecules in the mouse. Six heavy chain γG (γ2a), three heavy chain γH (γ2b) and four heavy chain γA determinants have been indentified. These determinants have been found to be closely linked in their genetic control. In addition, three other determinants which have thus far not been assigned to specific immunoglobulin molecules have also been indentified. The 16 determinants are present in the Fc region (constant region) of the heavy chain. In addition two γF alleles have been identified by the electrophoretic mobility of their Fc fragments. Nine heavy chain linkage groups have been identified; six in inbred strains and three additional ones in wild mice.

Immunoglobulin determinants in other regions of immunoglobulin molecules, namely the Fab region, have also been identified for different classes of immunoglobulins. These have been identified with homologous precipitating antisera prepared with specific myeloma proteins. These antisera identify myeloma specific determinants (MSD) or idiotypic determinants that are restricted to the Fab region of the myeloma protein used as the

immunogen.

Many of the myeloma proteins, specifically IgA's, have been found to have biologic activity, i.e., they have been shown to combine with Pneumococcus C polysaccharide, Salmonella polysaccharide, dextran, 2,4-dinitrophenyl group (DNP), 2,3,6-trinitrophenyl groups (TNP), etc.

Recently, 8 plasma cell tumors, induced independently in genetically similar inbred mice (BALB/c), produced IgA myeloma proteins all of which combined with phosphoryl choline, which is found in the C polysaccharides of Pneumococcus and Lactobacillus. Homologous antisera prepared to these 8 functionally similar myeloma proteins showed that five of these (M299, H8, T15, S63 and S107) have a common antigenic determinant which was found to be on the Fab portions of the respective myeloma proteins. This common antigenic determinant was not found on the light or heavy chains alone but was only present on the Fab or on preparations of recombined light and heavy chains from the respective myeloma proteins indicating that this was a conformational determinant derived from the interaction of the light and heavy chains. Sequence studies of the amino terminus of the light chains of T15 and H8 indicate that the first 21 residues of both proteins are identical.

Homologous antisera to the three remaining functionally similar proteins (M167, M511 and M603) identified myeloma specific determinants that so far are unique to each separate myeloma protein used for immunization. Amino acid sequence studies of the light chain of two of these (M167 and M603) showed that there were differences between these two proteins and T15 and H8, and between each other, beginning with the 9th residue.

Significance to Biomedical Research and the Program of the Institute:

It is difficult to study the genetic control of the diversity of antibody using normal antibodies since these are mixtures of many types of molecules. Myeloma proteins are pure molecular forms of immunoglobulins and these can be obtained by experimentally inducing plasma cell tumors in some strains of genetically defined inbred mice. In addition many of these proteins have been found to have antibody activity. Immunoglobulin determinants on both the common and variable regions of the heavy chains as well as conformational determinants involved in the antibody site provide tools to study the genetic control of the variability of antibodies. The finding of 5 identical myeloma proteins appears to indicate repetition of genetic mechanisms that control diversity of antibody production.

Project Subtitle II: Genetics of V-Regions

Project Description:

Objectives:

Phosphorylcholine binding is one of the activities most frequently found among the mouse myeloma proteins. Thirteen myeloma proteins with phos-

orylcholine binding have been found among IgA and IgG3 myeloma proteins induced in BALB/c. The phosphorylcholine proteins have been characterized by structural and antigenic (idiotypic) studies. Four of the available phosphorylcholine proteins have been shown to have the same antigenic (idiotypic) specificities. Recently it was found that normal BALB/c serum contained an antigen similar to S63-T15 idiotype whereas normal serum from C57BL mice lacked this antigen. We determined that there was a genetic difference (gene) between BALB/c and C57BL that controls the formation of the proteins (idiotypes) of the S63-T15 group. Characterization of this genetic control is presently being done.

Methods Employed:

Individual specificities of 6 myeloma proteins (a G3 myeloma protein and five IgA myeloma protein) all of BALB/c origin and binding phosphorylcholine were characterized by comparing their individual antigenic specifidities using the highly sensitive method of inhibition of hemagglutination. In addition normal serum and immune sera from different inbred strains, immunoglobulin congenic strains and immunoglobulin recombinant strains prepared by immunization with rough strain of pneumococcus (R36) were examined for the S63-T15 idiotype. BALB/c, C57BL, Bailey's recombinants [ (CxBd), (CxBe), (CxBg), (CxBi), (CxBj) and (CxBk)] and congenic $CB_{14}$ and $CBB_{16}$ were treated in this manner.

Major Findings:

Normal sera (pre-immune) and anti-R36 immune sera from BALB/c and from recombinants CxBg and CxBj carried the S63-T15 idiotype. All of these strains have the $IgC_H$ of BALB/c. Normal sera of CxBd, CxBe, CxBh, CxBi and CxBk which contain the $IgC_H$ of C57BL did not possess the S63-T15 antigenic determinants but after immunization with R36 it was present in some mice.

Both normal and anti-R36 antisera from CB14 and CB16 congenic st strains also failed to exhibit the S63-T15 antigen. These congenic mice were derived from backcrosses of C57BL/BALB/c heterozygotes for 14-16 generations respectively always selecting for the $IgC_H$ of the C57BL and then made homozygous for 5 generations. Presumably all these strains have the BALB/c genes except for the $IgC_H$ locus which is of C57BL origin.

Significance to Biomedical Research and the Program of the Institute:

The identification of genes controlling immunoglobulin variable region determinants permits studies of mechanisms of antibody diversity and formation of immunoglobulin molecules.

Project Subtitle III: Clonal Nature of the Immune Response to Phosphorylcholine

Project Description:

Objectives:

Characterize normal anti-phosphorylcholine antibodies with anti-idiotype antisera prepared with BALB/c IgA myeloma proteins TEPC 15, HOPC 8, MOPC 603, MOPC 511 and MOPC 167 respectively, all of which bind phosphorylcholine.

Methods Employed:

BALB/c and A/J mice were immunized with $10^8$ D. pneumoniae organisms (R36). After one or more injections the characteristics of the phosphorylcholine (PC) specific antigen binding cell (ABC) and plaque forming cell (PFC) response was assessed by inhibition of PC and its analogues as well as by antisera specific for idiotypic determinants of 4 different PC-binding BALB/c myeloma proteins (H8, M603, M511 and M167).

Major Findings:

The frequency of ABC, the precursors of PFC increase 100-fold upon primary immunization.

The receptors on the ABC from both immune and non-immune animals are IgM molecules specific for PC, and ABC from immune BALB/c but not A/J possess the idiotypic specificity of H8. During primary and secondary responses, the amount of PC which blocked 50% of PFC remained unchanged indicating a lack of "maturation" of affinity. The relative binding of PC and its analogues by IgM antibody also remained unchanged and was similar to H8. The PFC in BALB/c but not A/J mice were blocked by anti-H8, none of the other anti-idiotype antisera (anti-603, anti-511 and anti-167) blocked the PFC of either strain.

HI tests with normal and anti pneumococcus immune sera from A/J and BALB/c mice showed that H8 idiotypic antigen was present in BALB/c serum but absent in A/J serum.

Significance to Biomedical Research and the Program of the Institute:

These data indicate the monoclonal nature of the immune response to phosphorylcholine and promotes an insight into the diversity of antibody formation.

Serial No. NIAID - 148
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Genetic Control of Antibody Responses

Previous Serial No.: None

Principal Investigator: Miss Rose Lieberman

Other Investigators: Project Subtitle I: Dr. W.E. Paul, LI, NIAID
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Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 1 | | | 1 |
| Professional | 1/2 | | | 1/2 |
| Other | 1/2 | | | 1/2 |

Project Subtitle I: Genetic Control of Antibody Responses to Myeloma Proteins of Mice

Project Description:

Objectives:

Immunoglobulins, specifically myeloma proteins of BALB/c origin, comprise a group of antigens that are most useful in the identification and linkage analyses of genes controlling the immune response. An immune response gene IrIgA (M467) which controls the immune response to BALB/c myeloma protein MOPC 467 was found linked to the H-2 histocompatibility locus. Chromosome mapping showed that the IrIgA (M467) gene was located to the left of (centromeric to) the Ss-Slp gene(s). Since Ir genes appear to play a major role in susceptibility to diseases we continued our investigations on the genetic control of immune responses to other classes of immunoglobulins (myeloma proteins), specifically IgG ($\gamma_{2a}$) MOPC 173 and

IgH ($\gamma_{2b}$) MOPC 195 myeloma proteins of BALB/c mice.

Methods Employed:

MOPC 173 ($\gamma_{2a}$) and MOPC 195 ($\gamma_{2b}$) myeloma proteins, both of BALB/c origin, were separately used to immunize inbred, B10 congenic and B10.A recombinant strains of mice. The immune response of these mice was determined by titration by passive hemagglutination of antibodies to allotypic determinants present on the heavy chain constant region ($IgC_H$) and to idiotypic specificities present on the Fab segment and specific for the myeloma protein.

By selecting the appropriate congenic and recombinant strains to determine the immune response to these myeloma proteins, the genetics of the Ir genes involved could be ascertained.

Major Findings:

Four Ir genes have been identified and mapped on the H-2 chromosome. Ir-IgA (M467) is to the left of (centromeric to) the Ss-Slp genes.

Ir-IgG (M173) is to the right of the K locus and is located between the Ir-IgA and the Ss-Slp.

The Ir-IgH (M195)-1 locus is to the right of the Ss-Slp locus. It has not yet been determined whether this locus is to the left or right of the D gene or is equivalent to the D gene.

Ir-IgH (M195)-2 is to the left of Ss-Slp but so far cannot be separated from the Ir-IgG (M173) locus.

Significance to Biomedical Research and the Program of the Institute:

Ir genes are important in understanding of the role of heredity in susceptibility and resistance to disease. In addition, Ir genes also serve as genetic markers to study gene products at the cellular level.

The studies carried out here are the first to establish the existence of multiple, independent Ir loci within H-2. In particular, Ir-IgH (M195)-1 is the first Ir gene to be located in the region of H-2 to the right of Ss-Slp. This suggests that very large numbers of Ir genes may exist.

Proposed Course of the Project:

Attempts to more precisely locate each of these Ir genes and several additional ones now under study and to identify the precise antigenic determinants for which they are specific are planned.

Project Subtitle II: Genetic Factors Controlling Antibody Response and Immunoglobulin Synthesis

Objectives:

Backcross and F2 progeny of mice that had been selected for "high" and "low" antibody synthesis for twenty generations were analyzed for their immune response to sheep red blood cells (SRBC) to determine the genetic factors controlling this response. In this way some insight as to the number and types of genes concerned with the control of immunoglobulin synthesis could be determined.

Methods Employed:

Backcross and F2 progeny were bred from 2 separate lines of random bred Swiss mice that had been bred selectively for their agglutinin response to sheep and pigeon erythrocytes to obtain "high" and "low" responder lines for these antigens. The "lows" were bred to the "highs" to yield $F_1$ animals. They were then backcrossed to either "high" or "low". The F2 were derived from crosses of (high and low) $F_1$ mice. All the progeny were immunized with SRBC alternated with pigeon RBC and their agglutinin titers determined 5 and 17 days after immunization. Concentrations of immunoglobulins in the sera were determined 10-11 days after immunization.

Each mouse was typed for immunoglobulin allotypic determinants to determine their phenotypes.

Major Findings:

The immune response to SRBC in $F_1$, $F_2$ and backcross progeny of mice genetically selected for high and low antibody synthesis indicated that this response is controlled by a gene linked to the immunoglobulin heavy chain of the mouse. Mice homozygous for the phenotype of the high line produce significantly higher titers of antibody than mice homozygous for the low line.

However, the control of the immune response was clearly multigenic since heterozygous mice of the same H-chain phenotype resulting from 2 different types of backcrosses (high versus $F_1$ and low versus $F_1$) had very different immune responses. Immunoglobulin levels in the same progeny showed no linkage to the immunoglobulin allotype.

Significance to Biomedical Research and the Program of the Institute:

The genetic control of the immune response and antibody syntheses is concerned in understanding of resistance and susceptibility to diseases. This system may provide a model for analysis of some forms of hypogammaglobulinemia and hyporesponsiveness.

Publications:

Lieberman, R., Paul, W.E., Humphrey, W., Jr. and Stimpfling, J.H.: Genetic Control of Immune Responsiveness to IgG ($\gamma$2a) and IgA Myeloma Proteins. In McDevitt, H.O. and Landy, M. (ed.): Genetic Control of Immune Responsiveness. New York, N.Y., Academic Press, 1973, 117-122.

Lieberman, R., Stiffel, C., Asofsky, R., Mouton, D., Biozzi, G., and Benacerraf, B.: Genetic Factors Controlling Anti-Sheep Erythrocyte Antibody Response and Immunoglobulin Synthesis in Backcross and F2 Progeny of Mice Genetically Selected for "High" or "Low" Antibody Synthesis. J. Exp. Med. 136: 790-798, 1972.

Lieberman, R. and Humphrey, W., Jr.: Association of H-2 types with genetic control of immune responsiveness to IgG ($\gamma$2a) allotypes in the mouse. J. Exp. Med. 136: 1222-1230, 1972.

Lieberman, R., Paul, W.E., Humphrey, W., Jr. and Stimpfling, J.H.: H-2 linked immune response (Ir) genes. Independent loci for Ir-IgG and Ir-IgA genes. J. Exp. Med. 136: 1231-1240, 1972.

Lieberman, R. and Paul, W.E.: Genetic Control of Antibody Responses to Myeloma Proteins of Mice. In Warner, N. (ed.): Contemporary Topics in Immunobiology. New York, N.Y., Plenum Publishing Co., 1973, in press.

Serial No. NIAID - 149
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structural and Activity Studies on Immunologically Important Cells and Proteins.

Previous Serial Number: None

Principal Investigator: Dr. Myron J. Waxdal

Other Investigators: Project Subtitle II: Dr. Rose Mage, LI, NIAID
Dr. Salvatore Carta, LI, NIAID
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Project Subtitle IV: Ms. Virgina Aandahl, DCRT
Mr. LeRoy Brown, DCRT
Mr. Bob Martino, DCRT
Mr. Dave Songco, DCRT

Cooperating Units: Project Subtitle I: Dr. G.M. Edelman, The Rockefeller University, New York, N. Y.
Project Subtitle III: Dr. William Awad, School of Medicine, University of Miami, Coral Gables, Florida

Man Years:

| | NIAID Lab. Staff | Other NIH Personnel Assigned to Project | Guest Workers | TOTAL |
|---|---|---|---|---|
| Total | 2 9/12 | 1 9/12 | 3/12 | 4 9/12 |
| Professional | 1 3/12 | 1 9/12 | 3/12 | 3 3/12 |
| Other | 1 6/12 | - | - | 1 6/12 |

Project Subtitle I: Lectins and Their Interaction With Cells

Project Description:

Objectives:

1. To investigate the structures of certain peculiar lectins and their mode of interaction with cellular receptors.
2. To investigate the cell surface, receptors, and the initiation of events, such as mitogenesis, arising from interactions with these lectins.

Methods Employed:

Salt extracts of Phytolacca americana (pokeweed) roots were fractionated by solubility in high and low salt concentrations, hydroxylapatite chromatography and gel filtration. Five lectins were isolated by this procedure and were characterized by the usual physiochemical techniques establishing their molecular weights, number of polypeptide chains, subunit composition, amino acid composition and the availability of the terminal residues for identification. Biological characterization included their ability to agglutinate different types of human erythrocytes and ability to induce mitogenesis in murine splenocytes. The number of cellular receptors for two of these lectins was determined by the binding of the 125-I-labeled lectins.

Studies with Dr. Edelman at the Rockefeller University on the covalent and three dimensional studies of concanavalin A were completed using the Dansyl-Edman procedure to sequence peptides isolated after digestion of the CNBr fragments with proteolytic enzymes. The electron density maps were prepared from data collected by standard photographic techniques and the phases were calculated by the method of multiple isomorphous replacement.

Major Findings:

Five lectins were isolated from Phytolacca americana and designated Pa-1 through Pa-5. One of these (Pa-2) corresponded to the previously known pokeweed mitogen. The remaining four lectins were previously unreported. The amino acid composition of Pa-3, Pa-4, and Pa-5 resembled that of Pa-2 in that four residues account for 60% or more of the total residues in each protein. These four lectins also contain abnormally high amounts of cystine. In contrast, the amino acid composition of Pa-1, the largest lectin isolated, differed from that of the other four proteins and contained much lower amounts of cystine. Each of these proteins was comprised of a single polypeptide chain with a blocked $NH_2$- terminus. The lectins appear to be monomers under physiological conditions.

Hemagglutination studies have shown there is no correlation between blood type and agglutinability by these lectins. Four of the five proteins are hemagglutins. The 5th (Pa-4) is not a hemagglutin and inhibits hemagglutination by Pa-2, Pa-3, and Pa-5 but not Pa-1. Binding studies indicate that Pa-2 and Pa-4 bind to the same receptors and with the same avidity. These results suggest that Pa-2, Pa-3, Pa-4 and Pa-5 all bind to the same receptor. The binding of less than $10^3$ molecules of Pa-2 per erythrocyte is sufficient to cause agglutination. This finding suggests that a cell surface alteration may take place upon binding of Pa-2, and this alteration leads to the agglutination.

All of the five lectins are mitogenic for murine splenocytes. Lectins Pa-1, Pa-2 and Pa-3, which were the most active hemagglutins were also the most active mitogens. Pa-4 and Pa-5 were active at higher concentrations, and caused less uptake of $^3$H-thymidine than the more potent mitogens. Binding studies indicated that at the same concentration equal amounts of Pa-2 and

Pa-4 were bound to the bound to the splenocyte, as was the case with erythrocytes, but with different mitogenic response. Unlike the hemagglutination studies, in which Pa-4 acted as an inhibitor, Pa-4 did not inhibit mitogenesis by Pa-2 or any of the other lectins.

Unlike other soluble mitogens, which are mitogenic for either B cells or T cells, the pokeweed mitogens are active for both classes of lymphoid cells and are active over an unusually wide concentration range.

The amino acid sequence of concanavalin A, determined chemically, contains 238 residues. Interpretation of the X-ray crystallographic data at 2A resolution indicates that the predominant structural element in Con A is extended polypeptide chain arranged in two antiparallel pleated sheets or β-structure. Residues not included in the β-structure were arranged in random coils. The positions at which $Mn^{+2}$, $Ca^{+2}$ and saccharide bind to the protein have been tentatively assigned.

Significance to Biomedical Research and the Program of the Institute:

The physiochemical and binding similarities between the pokeweed lectins, especially Pa-2 and Pa-4, as opposed to their different hemagglutination and mitogenic properties appears to provide an unusual system for the study of the cell membrane and lectin induced mitogenesis. The use of these lectins and others as probes of the cell surface will bring to light differences and similarities in the triggering of mitogenesis and antibody production by T and B cells. These probes will also provide new information on the differences of the cell surface during differentiation, mitosis, antibody production, and transformation. Relationships between cell surface interactions and control of these events will also come to light.

Concanavalin A is the first lectin and macromolecular mitogen whose structure has been determined. It is a subunit protein, and physiochemical experiments interpreted in terms of its three-dimensional structure should provide insights into the stabilization of the protein by metal irons, subunit equilibrium and cooperativity of saccharide and metal binding. Furthermore, this structure provides a starting point for studying the detail interaction of this mitogen and the cellular receptor.

Proposed Course of Project:

The pokeweed system, apparently non-aggregating single chain molecules, may provide better fidelity in the future study of the cell surface than with other current lectins. These studies will provide information as to the type, distribution and interaction of cellular receptors for these and other lectins. Structural studies of the lectins and cellular receptors will shed light on the type of interactions necessary to stimulate mitogenesis and cause cell agglutination. The effect of these interactions on growth rate, differentiation, and viability will also be investigated.

Project Subtitle II: Structural Studies of Immunoglobulins

Project Description:

Objectives:

1. To study the salient features of the antibody combining site and the production and structure of different antibodies which recognize the same antigen.

Methods Employed:

Certain of the rabbits in our genetically defined population respond to immunological challenge by producing a limited number of antibody types against the immunizing agent. The sera of one of these animals has been fractionated by salt precipitation and column chromatography using ion exchange resins. The resulting purified antibodies have been subjected to physiochemical characterizations including isoelectric focusing, separation of light and heavy chains, micropeptide maps, and amino acid analysis of all their structural components.

Major Findings:

Isoelectric focusing of the immunoglobulin isolated from one of the immunized rabbits indicated the presence of three major antibodies. These antibodies were isolated by column chromatography and their light and heavy chains prepared. The preliminary evidence, based upon amino acid analysis and micropeptide maps, suggests that the light chains of these three antibodies may be identical and that the differences lie in the heavy chain.

Significance to Biomedical Research and the Program of the Institute:

These studies are aimed at elucidating the structure of the combining site of antibodies. The redundant and compensatory features, by which different antibodies are able to recognize the same antigen, will increase our detailed knowledge of the combining site. An understanding of these protein structures will help to define the genetic mechanisms, and their control, which lead to the humoral immune response.

Proposed Course of Project:

The structures of these three antibodies will be further studied to determine the regions of similarities and differences between these antibodies. The structural differences will be localized and defined.

Project Subtitle III: Structure and Activity of Proteins of Note

Project Description:

Objectives:

To investigate the structure and activity of proteins possessing a special property to make them of current interest. In this instance, a chymotrypsin-like enzyme had been previously isolated from the K-1 strain of Streptomyces griseus. This protein was both active and stable in 6M guanidine-HCL. The salient structural features resulting in this remarkable stability, and the use of this enzyme under conditions where other proteins are denatured are under investigation.

Methods Employed:

The purified protein has been subjected directly to sequence analysis using the Beckman Model 890C sequenator. The protein has also been cleaved into fragments by reaction with cyanogen bromide. The presumptive active seryl residue has been labeled with $^{32}P$ di-isopropylphosphorofluoridate and the radioactive tryptic peptide isolated. The protein, fragments and peptides in this study were fractionated by column chromatography and gel filtration techniques.

Major Findings:

The amino acid composition of this protein indicated that it contained two methionyl residues and had two disulfide bonds. The protein was cleaved into three fragments by CNBr. These fragments were isolated by gel filtration techniques and accounted for the entire protein molecule, on the basis of molecular weights and amino acid compositions. The fragments were ordered by comparison of their $NH_2$-terminal sequences to that of the intact molecule, and the specificity of the CNBr reaction. The three fragments have been named $F_1$, $F_2$ and $F_3$ in order of their tentative arrangement in the polypeptide chain. $F_1$, the largest fragment, contains one of the disulfide bonds. $F_3$, the second largest fragment, contains the other disulfide bond and the radioactively labeled active seryl residue.

$F_3$ was reduced and alkylated, then digested with trypsin and the expected three peptides isolated ($T_1$, $T_2$, $T_3$). Direct sequence analysis on $F_3$ overlapped the first two of these peptides and established the order as $T_1$-$T_2$-$T_3$. Peptides $T_2$ and $T_3$ contained CM-cysteine and hence these regions were joined by a disulfide bond in the intact protein. $T_2$ also contained the labeled active seryl residue.

Sequence analysis of the intact protein, fragments, and peptides show a high degree of homology between this protein and functionally related serine proteases produced by fungi and bacteria. Furthermore, homology was also evident between this protein and bovine chymotrypsin.

Significance to Biomedical Research and the Program of the Institute:

The unusual stability of this protein in denaturing solvents resides in the folding of its single polypeptide chain and the non-covalent interactions between various portions of the chain. The protein contains only two disulfide bonds, which should be insufficient to confer such stability. A knowledge of how this stability is maintained will be useful in understanding one of the last fundamental questions in protein structure: How primary structure determines tertiary structure and activity. Furthermore, like the immunoglobulins in which grossly different antibodies can have the same specificity, enzymes from widely separated organisms, vis., fungi and cow, have the same specificity. The gross structures of these enyzmes are different, but certain homologies have arisen by convergent evolution. These common features in the active site are responsible for the common specificity. Study of this system, like that of antibodies, will shed light on redundancy and compensation in protein structure.

Proposed Course of Project:

The structural studies, including sequence analysis, will be continued on this enzyme. The stability of this enzyme will make it useful as a tool in studying the conformation and stability of other proteins in denaturing solvents. This enzyme will also be used to digest insoluble peptides, the "core" of other proteins under structural investigation.

Project Subtitle IV: Laboratory Automation and Data Processing

Project Description:

Objectives:

To achieve maximal utilization of several mechanized analytical instruments utilized to pursue the objectives of LI/NIAID by automatic data acquisition, data reduction and calculation, data display and instrument monitoring. To develop new automated procedures for structural studies on cells and proteins, which will reduce the time, effort, and mistakes inherent in conventional procedures.

Methods Employed:

A dedicated digital mini-computer will be interfaced via digital and analogue hardware both to receive information from and transfer information to several laboratory analytical instruments. The incoming data will be acquired and processed under real time program control. The raw data will be reduced and filed in the computers disc storage, or transferred via data lines to the PDP-10 time sharing computer in DCRT. This data will be used to make online decisions for the control of the laboratory instrument or held for further processing under the operators control. The computer will control automated instruments to provide the most efficient of these instruments and allow the development of new procedures, impractical by conventional techniques.

Major Findings:

Proposals for the computer system were received from several companies and evaluated. The contract was awarded, but upon arrival the computer did not successfully meet the specifications and the company was forced to remove the computer. The contract has been re-awarded and a PDP-11 with the necessary peripheral equipment is being acquired from Digital Equipment Company and will be installed in May 1973. This minicomputer, and the dataphone link to the PDP-10, will provide the necessary computational hardware to achieve the objectives of this project.

The necessary digital and analogue hardware is being designed, developed and constructed to provide interfacing between the PDP-11 and the laboratory instruments.

The specifications and overall concept of the software for the immediate future of this project has been completed. Portions of the necessary modular programs have been coded and are awaiting the arrival of the computer.

By June 1973 the first laboratory instrument, a Gilford spectrophotometer will be interfaced to the computer and in operation. This instrument will monitor the effluent from four chromatographic columns used to separate proteins and peptides. The computer will acquire the effluent absorbancy from these columns on elapsed time basis and correlate this data with the current fraction being collected in one of four fraction collectors. Upon operator request, the computer will supply a concentration or absorbancy vs fraction number graph for any of the four columns. Further processing and calculations will also be available to the operator.

Significance to Biomedical Research and the Program of the Institute:

The automation of data acquisition and processing and of instrument control will markedly increase the productivity of the instruments and the investigators using them. Much of the redundant and mistake prone drudgery of manual methods will be alleviated, freeing laboratory personnel to make better use of their training and experience for higher duties. Furthermore, new procedures will be developed and projects accomplished which are impractical conventionally but become possible using automated techniques and data processing.

Proposed Course of Project:

In the next year a Beckman Amino Acid Analyzer and a Hewlett-Packard Gas Liquid Chromatograph will be interfaced to the computer and automated. This will provide on-line identification and quantitation of the amino acid analyzer of protein and peptides. In addition, the PTH-amino acids obtained from sequence studies will be identified and quantitated automatically. Reports of these analyses will be automatically calculated and prepared by the computer.

Publications:

Edelman, G.M., Cunningham, B.A., Recke, G.N. Jr., Becker, J.W., Waxdal, M.J. and Wang, J.L.: The covalent and three dimensional structure of concanavalin A. Proc. Nat. Acad. Sci. 69: 2580, 1972.

ROCKY MOUNTAIN LABORATORY

HAMILTON, MONTANA

July 1, 1972 through June 30, 1973

Summary ..... 1

170 - Office of the Director.....11

171 - Rickettsiae and Rickettsial Infections.....12

173 - Selected Zoonoses of Regional Importance.....21

174 - Systematics and Vector Relationships of Certain Parasitic Arthropods.....28

175 - Allergy, Its Mechanisms and Role in Disease.....35

177 - Immune Prophylaxis of Mycobacterial Infections.....43

178 - Structure and Biologic Activity of Endotoxins.....49

179 - The Role of Morphological Elements of Microorganisms in Cancer Immunotherapy and Related Phenomena.....53

180 - The Encephalitides.....60

181 - Tick-Borne Disease Agents.....63

182 - The Epidemiology of Human Infections of Special Interest.....69

183 - Viruses as a Probable Cause of Certain Chronic Diseases of Man.....73

184 - Biological Properties of Bordetella pertussis Antigens.....81

185 - Agents of the Psittacosis-Lymphogranuloma-Trachoma Group (Chlamydia).....85

186 - The Biology of Microbial Agents in Arthropods.....90

187 - Host-Ectoparasite Relationships.....96

188 - Vector-Pathogen Relationships.....98

PHS-NIH

Summary Statement

Rocky Mountain Laboratory

Hamilton, Montana

July 1, 1972 through June 30, 1973

During the past year the scientific staff continued to pursue their research programs with vigor and enthusiasm, despite the uncertainties of future support and further reductions in personnel. Only one scientist could not be supported with adequate technical assistance for sustaining a productive program, and this problem will be resolved in the near future. Reorganization of the laboratory from 9 to 7 research sections enhanced productivity and stimulated more effective supervision of some programs by section heads. During the year Dr. John Bell, Dr. Cora Owen, and 14 supporting staff retired or resigned, and one was lost through death. Although 7 positions were lost, the authority to use 10 of these allowed us to strengthen some of our more important programs. Dr. Francis Abinanti will collaborate with Dr. Ribi on tumor immunotherapy. The additions of Drs. Chesebro, Portis, and Bloom to our Allergy and Immunology Section and the return of Dr. Lodmell has stimulated more extensive collaboration with staff of the Slow Viral Diseases Section. As explained in the text of the annual report, a number of promising projects have been initiated on the immunologic aspects of slow viral infections. Because of the nature of these diseases, sufficient time has not elapsed for definite findings, but results should be forthcoming during the next year.

Our major research programs continue to be oriented toward the microbiology, immunology, and control of animal-borne, arthropodborne, and certain chronic progressive viral and bacterial diseases, and toward more basic aspects of bio-immunologic response to antigens and microbial constituents. The greater emphasis on tumor immunotherapy is our only significant program change. Selected research highlights are presented in the following sections.

Rickettsioses

In the course of investigations now completed on the ecology of spotted fever rickettsiae in Tennessee, CF antibodies to this organism were found in 8 of 23 species of medium-sized mammals. Of 666 serums, 54 were positive, with higher rates among rabbits, raccoons, and woodchucks. About 5% of Dermacentor andersoni were infected, but the organism could not be recovered from any of 545 Amblyomma americanum, which has long been considered a vector of this agent. In a related study near Fayetteville, Arkansas, almost 9% of D. variabilis were infected, but again, none of 482 A. americanum were infected. (Burgdorfer)

A rickettsialike organism related by immunofluorescence to the spotted fever group was detected in A. americanum taken in both Tennessee and Alabama. Its fine structure indistinguishable from pathogenic rickettsiae, this organism was found in moderate quantities in all tick organs and was passed transovarially to 100% of filial ticks. It failed to grow in fertile hens' eggs, was nonpathogenic for voles and guinea pigs, but could be cultivated on primary chick embryo tissue cultures. Another rickettsia-like microorganism related by immunofluorescence to both spotted fever and typhus groups was found in 7 of 102 Ixodes scapularis from Arkansas. All tick tissues were heavily infected, but it also was noninfectious for laboratory animals. Further studies on these organisms will be made to determine their relationship to established species of rickettsiae. (Burgdorfer, Brinton)

To evaluate the effect of spotted fever rickettsiae on spermatogenesis in D. andersoni we obtained baseline data on the process in normal ticks. In unfed males, spermatogonia were partitioned into cysts by a simple squamous epithelium. Within 24 hours after tick attachment to a host, those in the posterior region of the paired gonads enlarged synchronously to form primary spermatocytes. All germ cells in a given cyst were connected by intercellular bridges and development was synchronous through completion of both meiotic divisions. (Brinton)

Sufficient experience with the microagglutination (MA) test has now been obtained to evaluate its use in rickettsial serology. It is sensitive and rickettsia-specific but has limited application. In a study in which serologic methods were compared in tests of 1,628 sera taken during the last 10 years from RML employees vaccinated with phase I Q fever vaccine, the correlation between results of phase II and radioisotope precipitation (RIP) tests was excellent, but that between phase I MA and RIP tests was not at RIP titers of 1:3200 and lower. In this study, the CF test was relatively insensitive, compared to MA and RIP tests. Sera from patients affected with typhus or spotted fever reacted more specifically in the CF test than in the MA test; extensive cross reactions by the MA test were seen between typhus antigens and spotted fever group antibodies, and vice versa. Similar cross reactions were seen by immunofluorescence tests, and the sharing of common antigens by spotted fever and typhus groups is strongly suggested. Since most of the serologic data relating to the supposed animal reservoir of epidemic typhus in Africa was based on agglutination tests, and since ticks parasitizing livestock there are commonly infected with spotted fever rickettsiae, one questions whether spotted fever infections were erroneously interpreted to be Rickettsia prowazeki infections. (Ormsbee)

It has been postulated that Coxiella burneti found naturally in animals is always in phase I. To determine whether a phase II could revert under host pressure to phase I, we followed the infection induced in guinea pigs inoculated with a pure cloned phase II in the 90th egg passage. Phase I antibodies could not be detected and the organism could not be maintained through more than two consecutive passages. The behavior of this culture in animals was similar to that of the 1/M-44 strain used by the U.S.S.R. as a live vaccine in man. (Ormsbee)

Further investigations were made on improving the quality of rickettsial vaccines. Purified rickettsial particulate antigens have now been prepared from 7 species of rickettsiae by centrifuging organisms that had been grown in fertile hens' eggs or tissue culture in a reorienting density gradient rotor. The yield per gram of tissue of mouse L cell culture was 10 times that obtained in fertile hens' eggs. The protection afforded guinea pigs vaccinated with these purified particulate spotted fever antigens was about 10-fold greater than that induced by a comparable amount of commercial yolk sac vaccine. (Anacker, Gerloff)

Tick-Borne Viruses

Continued investigations have demonstrated the importance of Ixodes uriae as a vector of at least 5 viruses (all presumably seabird) along the west coast of North America, from Alaska to southern Oregon. So far, this species has been found infected with Yaquina Head, Tuleniy, "86" (Sakhalin), Uukuniemi, and Great Island virus, which was previously recovered from this tick species in Newfoundland. By testing individual ticks or small pools, we showed that about 6% were infected with "86" virus and that this virus was likely transmitted transtadially and transovarially. Even higher infection rates were observed among Argas cooleyi, a parasite of cliff swallows, taken in New Mexico. Some colonies were free of virus, whereas others had tick infection rates of 30%, with either Sixgun City virus or sapphire II virus. The public health problem of these viruses is unknown, although some related viruses in other parts of the world are associated with human disease. However, in view of the prevalence of these ticks, their distribution, and high virus infection rates, the potential health problem cannot be ignored, if the ecosystem of these ticks is invaded by man. (Clifford, Yunker, Thomas)

Colorado Tick Fever (CTF)

Because of the prevalence of this disease and the need for better diagnostic methods, the fluorescent antibody (FA) technique was evaluated for both the detection of virus in red cells and the immune response to it. CTF virus was isolated in mice from 34 of 50 human blood samples that had been stored for 3 to 18 months. By the FA method, viral antigen was readily detected in erythrocytes of samples taken after the 4th day of illness, but only 4 of 17 mouse-positive samples taken during the first 4 days were FA positive. In a laboratory-acquired case, viral antigen was detected in erythrocytes from the 6th day to the 124th day after exposure. With an indirect FA assay, using CTF (Florio) infected Vero cells as antigen, antibodies were detected in 34% of 186 serum samples in titers from 1:8 to 1:4096, whereas only 13% had antibodies detectable by CF test. Thus, the FA method is a more sensitive assay for antibodies and, after the 4th day, equally as sensitive for detecting virus as the mouse inoculation technique. The long periods during which parasitized erythrocytes circulate raise questions about the importance of this health problem in the Rocky Mountain area. Conceivably, CTF could be a problem to recipients of whole blood taken from persons who had recently had the disease. (Philip, Casper, Thomas, Yunker)

## Vector-Pathogen Relationships

Various parameters of the growth and behavior of arboviruses in arthropod cell cultures were investigated. Of 124 viruses tested, 30 induced plaques in Aedes albopictus mosquito cell monolayers. Plaquing ability reflected group relationships and vector class. In general, group A arboviruses and tick-borne virus failed to plaque, whereas almost all group B mosquito-borne viruses caused plaques. Of 20 arboviruses that caused plaques in A. albopictus monolayers, only 5 induced poorly defined plaques in Aedes w-albus cells. However, growth of Murutucu virus (group C), which failed to plaque in this cell line, was 1 1/2 logs higher than that reached in A. albopictus cells. Only spotted fever group rickettsiae formed plaques in A. albopictus cells but this group, typhus group, and Pope's rickettsia formed excellent plaques in Vero cells.

In continued efforts to establish an in vitro line of cells from ticks the most promising results to date were obtained with the serial cultivation, for up to 4 passages, of large numbers of hemocytes. Insight into the media modifications required for growth was gained, in part, from data from our PL-480 project with NAMRU-3. Up to 7 passages were made of tick hemocytes cultivated on mitotically inhibited feeder layers of mosquito cells, but since the effectiveness of mitomycin is eliminated at this passage level, the identity of the cells—whether tick, mosquito, or hybrid—becomes moot. (Yunker)

## Systematics of Arthropods

Significant progress has been made in curating the Bishopp tick collection transferred to RML in 1971. Over 2,900 lots containing thousands of specimens have been identified. Data from these are being programmed on punch tape for entry into the Smithsonian data retrieval system. About 400 collections of ticks from Nepal have been identified and a manuscript describing known and new species of Ixodes is in preparation. (Clifford, Keirans)

A preliminary investigation of the application of scanning electron microscopy to the study of medically important Acarina was made. Adult specimens of 4 species of closely related Ornithodoros ticks (capensis group), which are distinguishable only as larvae, were number-coded, divested of other identification, and photographed. The 4 species were clearly separated by distinguishing morphological features not seen under light microscopy. Further studies will be directed toward establishing limits of variation within a species. (Yunker, Clifford, Keirans)

About 150 species, nearly half of them new, were identified in a recently completed study of 20,000 chigger specimens from Venezuela. A sizeable report of this study will be in press soon. Comprehensive studies of the genus Eutrombicula of the Neotropical region and of the chiggers of Surinam are progressing slowly, but pertinent to this work, 5 manuscripts have been published and 2 are in press. A study of nearly 2,000 collections of mites formed the basis for 6 publications of new species or records. (Brennan, Yunker)

## Abortive Rabies and Immunoprophylaxis Against Rabies

Results of continued research on the mechanism of abortive rabies have shown that hyperthermia not only reduced mortality, as based on the number of mice showing clinical signs, but also increased the rate of abortive infection, which is a distinct phenomenon. As reported last year, the histamine sensitizing factor (HSF) of Bordetella pertussis also markedly increased the survival rate, presumably through an accelerated immune response. In limited trials, BCG also reduced the number of mice affected with patent rabies and increased the survival rate of those clinically affected.

In animals the appearance of rabies neutralizing antibodies in the CNS has been firmly associated with recovery from rabies. However, in exposed persons who develop encephalitis after treatment with vaccine, antibodies in the CNS may have resulted from a breach in the blood/brain barrier induced by a concomitant allergic encephalomyelitis. So far, we have been unable to breach this barrier in animals by purposely inducing experimental allergic encephalomyelitis. (J. F. Bell)

Bordetella pertussis extract (BPE) markedly accelerated immunogenesis when added to the relatively poor commercial duck embryo vaccine. Experiments with various purified fractions of this extract indicated a close correlation between enhancement and the HSF. If further studies on this acceleration and enhancement confirm initial findings, and if anergy to rabies vaccine in exposed persons or animals and other possible hazardous side effects can be confidently excluded, the use of BPE or pure HSF in the treatment of rabies-exposed patients is strongly indicated. Because of a number of serious problems and disadvantages, the current use of immune globulin, in conjunction with vaccine, is questioned. Hence the use of BPE to accelerate the immune response may be more effective and less dangerous. (Bell, Munoz)

## Psittacosis-Lymphogranuloma-Trachoma (PLT) Group

Much of the research on this project was oriented toward further evaluation of the micro-immunofluorescence (micro-IF) and radioisotope precipitation (RIP) tests for their ability to identify immune responses to PLT-group and type-specific antigens. Serums from 575 persons in 4 epidemiological categories were tested. Of 300 serums from VD patients in England affected with gonococcal and nongonococcal urethritis, 85% and 94% of those from whom chlamydiae were isolated were positive in micro-IF and RIP tests, respectively. Those in the chlamydia-negative group had positive rates of 55% and 82%. Antibody titers correlated with the recovery of chlamydiae, but the high reactivity rates were more impressive than the differences. In contrast to these high seropositive rates, those seen in 2 groups of children from Minnesota were much lower. Only 20% and 22% of children of 6.8 years average age were seropositive by RIP and micro-IF, respectively, and 15% of children of 1.8 years average age had antibodies detectable by both tests. Of 91 serums submitted to RML from patients thought to have a

zoonoses, 38% were positive and reactivity by age in this group corresponded to their expected patterns of sexual activity. There is little reason to question the specificity of these 2 tests and there is strong evidence for wide-spread distribution of PLT-group agents, especially among sexually promiscuous populations.

So far, over 800 serums have been tested by both methods and the correlation has been excellent in all study groups. The RIP test measures PLT-group antibody and is about 6-fold more sensitive than the micro-IF, particularly in tests of sera free of type-specific antibody. The micro-IF detects both group and type-specific antibody, which is chiefly associated with the IgM class. The presence of IgM also is associated with recent infection and with the isolation of chlamydiae from patients. (Philip, Gerloff)

Immunoprophylaxis Against Tuberculosis

One of the unexpected side products of our past research on BCG cell wall (CW) vaccine was the finding that the immunity induced by living BCG used in control mice and monkeys inoculated intravenously grossly exceeded that obtained by other routes. Although the IV route would be impractical for human use, the same immunity could possibly be achieved by the aerosol administration of BCG. For this reason, a collaborative study with the Tuberculosis Panel of the U.S.-Japan program was organized to compare the intravenous and aerosol routes with the intradermal route now being used for vaccination of man. At 3 months after aerosol challenge (5 months after vaccination), monkeys inoculated intravenously were completely protected, and those vaccinated with aerosolized BCG were nearly as well protected. In contrast, the extent of disease in those vaccinated by the intradermal route differed little from that seen in unvaccinated controls. Considering the close relationship between monkey and man, it would appear that the aerosol route should be used for vaccination of man.

The role of hypersensitivity in immunity against tuberculosis has long been a controversy. To date, the protection induced in mice given various BCG CW vaccines was correlated with the extent of the granulomatous response (GR) in the lungs. To determine whether protection was dependent upon the GR, we induced tolerance in mice by multiple injections of BCG CW in Tween saline and then vaccinated the tolerant animals with CW vaccine in oil droplets. Though these animals had a negative footpad test to PPD and had less GR than control vaccinates, the protection against aerosol challenge was even less than expected. In fact, some with extensive GR in their lungs were protected no better than unvaccinated controls. This finding suggests that specific immunity is required and that GR, possibly an index of hypersensitivity, is not necessarily associated with protection.

Some of the research on this project has been closely coordinated with our work concerning the role of mycobacterial fractions in tumor immunity. Two well-defined components of the cell wall were required to produce both GR in the lungs of mice and protection. Cell wall skeleton (CWS-I), a peptidoglycolipid remaining after organic solvent extraction, did not cause GR or induce protection in mice inoculated IV. Neither did $P_3$, a trehalose

ester obtained as a free lipid from wax D or cord factor. However, when the two were recombined and injected IV into mice, vigorous GR resulted and mice were protected. It is postulated that the CWS-I contains the specific immunogen and that the $P_3$ acts as an adjuvant. Some wax D preparations, which were reported to be immunogenic, were found to be contaminated with CWS-like material that provided the specific immunogen. (Meyer, Ribi, Azuma, RML; Barclay, AMA; Good, Hazleton Labs)

Adjuvant Action of Mycobacterial Cell Walls in the Immunotherapy of Tumors

Highly significant progress was made in the identification of the components of BCG that are involved in immunotherapy of tumors. The CWS-I remaining after a series of treatments with proteolytic enzymes and organic solvents was shown to be an insoluble polymer complex consisting of a long chain fatty acid (the mycolic acids), a polysaccharide of arabinose and galactose units, and a mucopeptide consisting of N-acetylglucosamine, N-glycolyl muramic acid, alanine, glutamic acid and diaminopimelic acid. This CWS-I did not protect mice against tuberculosis nor regress established tumors but did, even in small doses, produce systemic tumor immunity.

The free-lipid extract obtained by treatment of cell walls with proteolytic enzymes and organic solvents contained numerous components, most of which have not been identified. The $P_3$ fraction, studied to some extent last year, was identified as trehalose esterified to at least 3 structurally different mycolic acids. This substance was highly toxic for mice but, by itself, neither regressed nor suppressed tumors. However, when recombined with CWS-I, the activity of the original cell wall was restored both in protection against tuberculosis and regression of established tumors. Another mycolic ester (termed $P_4$) of higher polarity, whose activity was comparable to $P_3$, was regularly isolated from the "free lipid" extract. After these two substances were removed from the "free lipids", the material remaining at the top of chromatographic columns consisted chiefly of a mycolic acid-arabinogalactomannan-mucopeptide complex with structural features similar to CWS-I, but of lower molecular weight. This soluble peptidoglycolipid not only suppressed tumors but also regressed tumors, even though it lacked a detectable quantity of $P_3$. Further refinements in separation are being made to secure CWS-I fractions completely free of tuberculoprotein and consequently totally inert in eliciting a response to PPD. Theoretically, such a product would be more effective because the total immune response would be directed to the tumor specific antigen. Collaborative arrangements have been made with several investigators who are interested in testing BCG cell wall products in clinical trials in man. (Ribi, Meyer, Azuma, Abinanti)

Molecular Biology of Endotoxin (ET)

The reduction in support of this project the past year made it necessary to direct most research efforts toward the evaluation and standardization of the Limulus test. The recent discovery that the level of circulating endotoxin is of prognostic value in severe injury or disease has created a great demand for the test in clinical laboratories. Our ability to

detect as little as 8 picogram amounts of ET confirms the exquisite sensitivity reported by others; however, most preparations were clotted by 0.1 to 0.2 nanograms, which is about the same as a minimum pyrogenic dose for rabbits. The specificity of the Limulus test is equally outstanding. Polyriboinosinic-polyribocytidylic acid (poly I-C), which is chemically unrelated to ET, produces fever curves in rabbits superficially similar to that caused by ET. Poly I-C did clot Limulus lysate, but only in doses much larger than those required to produce fever in rabbits or to kill chick embryos. Procedures are being developed for packaging and distributing stable quantities of Limulus lysate as standards for use in clinical laboratories. (Milner)

## Bordetella Pertussis Antigens

Our persistent efforts to purify the histamine sensitizing factor (HSF) from Bordetella pertussis have finally been successful. The process involved extraction of acetone-treated cells in 1 M NaCl containing 0.05 M Na pyrophosphate, dialysis, fixation of extract to hydroxylapatite columns and elution therefrom with phosphate buffers of increasing molarity, acid precipitation, and a last step involving electrophoresis in agarose. The final product was fully active and contained no demonstrable antigens when tested with rabbit hyperimmune serums against the crude extract or the pure fraction. Fractions were tested as they were obtained in the purification process, and only those containing HSF stimulated leucocytosis, induced hypoglycemia and hypoproteinemia, served as an adjuvant in the production of experimental allergic encephalomyelitis, increased the production of reagenic antibodies in mice, and protected mice against infection with B. pertussis. However, correlation with the last parameter was on a qualitative basis only.

The physiologic action of HSF has not been fully explained, but a change in permeability of the vascular bed was shown to be a factor by monitoring the loss of $^{131}I$ HSA from the thigh muscles of mice treated with B. pertussis extract 3 days earlier. Treated mice lost 50% more labeled antigen than did untreated controls. Because of its diverse host reactivity, HSF must affect other body systems. The availability of the purified material should now enable clarification of its physical and chemical structure and its mode of action. (Munoz, Bergman)

## Synthesis and Activity of Ig Classes

Because of the unique brevity of the gestation period of the hamster, the neonatal development of its Ig system is of interest. $7S\gamma_2$ was regularly present on the day of birth, and $7S\gamma_1$ was occasionally found, but, at this time, both were derived from the dam's milk. Synthesis of $7S\gamma_2$ in lymph nodes and spleen began at 10 days of age, whereas synthesis of $7S\gamma_1$ began 10 days later and occurred only in lymph nodes. Synthesis of IgM and IgA began on the 5th and 8th day, respectively. Thus, the $7S\gamma_1$ was the slowest developing Ig and suggests its relatively recent evolution in the rodent Ig system. On the other hand, because the greatest relative increase in serum concentration after antigenic challenge occurs in the $7S\gamma_1$ class, this Ig must have the major role in immune responsiveness. (Coe)

Hamsters immunized with HEA in saline are known to produce mainly $7S_{\gamma 1}$ while those immunized with HEA in complete Freund's adjuvant produce both $7S_{\gamma 1}$ and $7S_{\gamma 2}$ types. When HEA $I^{131}$ was given the former, they eliminated about 40% of the antigen within 1 hour, whereas the latter eliminated 50% in 48 hours. Most hamsters in the latter group died within 2-4 hours after antigen administration. The sequestering of antigen in the lungs and liver and an acute pulmonary vasculitis observed suggest an immune complex disease as the cause of death. Animals lacking the $7S_{\gamma 2}$ (HEA saline) did not show this apparent immune complex disease. (Portis, Coe)

Immunology of Slow Viral Diseases

Tumor immunity in Friend virus leukemia (FVL) in mice is being studied to determine the relationship between the immune response to tumor-specific cell-surface antigens and recovery from this disease. Variations in susceptibility among strains of mice are thought to be controlled by a genetic trait closely linked to the major histocompatibility complex (H-2). Leukemic spleen cells from H-2 (d/d) mice, which rarely recover from FVL, have serologically detectable tumor antigens that decrease markedly during the course of disease. Similar antigens on leukemic spleen cells from H-2 (b/b) mice, which nearly always recover, do not decrease. Breeding experiments with mice that have proven genetic recombinants within the H-2 complex are being done in collaboration with Dr. Jack Stimpfling of the McLaughlin Research Institute, Great Falls, Montana, to determine which area of the H-2 complex controls the factors that affect recovery. (Chesebro)

A number of immunologically oriented projects have been initiated in collaboration with the Slow Viral Diseases Section. Immunoglobulins from mink 3 to 4 months postinfection with Aleutian disease virus have been covalently linked to Sepharose in an attempt to isolate this virus by immunoabsorption techniques. Conjugates have been made of horseradish peroxidase and rabbit antibodies to gamma globulin of mink, goat, and guinea pigs. With these reagents we should be able to demonstrate specific binding of globulin to virus or virus containing cells in various systems; preliminary attempts to demonstrate chronic pneumonitis virus grown in tissue culture have been encouraging. Mice and rabbits have been inoculated with scrapie infectious material complexed to a good immunogenic carrier molecule in an attempt to produce neutralizing or binding antibodies to the scrapie agent. Screening is being done for both binding antibodies and possible scrapie infectious immune complexes with the use of anti-immunoglobulin sera from other species. Hopefully, these projects will yield significant data during the next year. (Chesebro, Bloom, Munoz, Hadlow, Jackson, Race)

In vitro assays have been developed for measuring both humoral and cell-mediated immunity to herpes simplex virus infections in mice. A marked correlation between cytophilic and neutralizing antibody titers has been shown in the humoral immune response, and the immunological destruction of herpes-infected cells by immune lymphocytes has been demonstrated. In studies on the role of macrophages in immunity to herpes infection, we

showed that these cells prevented plaque formation and inhibited viral replication in virus-infected monolayers. A combination of macrophages, antiviral Ig, and complement was required to eliminate virus from an infected monolayer. (Lodmell)

Slow Viral Diseases

Additional virologic data support the thesis developed last year that the intestinal tract and associated lymph nodes are early sites of viral replication in natural scrapie, which manifests itself only after virus reaches the CNS. Whether disease represents the exception in a flock containing many sheep infected at the non-neural level can only be surmised. To resolve this question we are now testing key tissues of 15 clinically normal 4- to 6-year-old Suffolk sheep from high risk family lines.

Virologic data laboriously acquired during the past few years now indicate that encephalopathy (EN) in mink is similar to scrapie in goats—a dead-end infection without a regular exit of virus from the host. The limitation of infection to the lymph nodes and CNS in mink explains the failure of the disease to become established in herds of mink. The relationship of EN virus to scrapie has not been fully defined. There is strong epidemiological evidence that the natural disease was acquired by ingestion of scrapie-infected animal products. When suspensions of brain from one Cheviot and two Suffolk sheep were inoculated into pastel mink, only mink that received inoculum from one of the Suffolk developed EN. Whether this result reflects differences in virus titer or host-influenced variations in pathogenicity is not known. So far, mice have been refractory to EN virus, even after one passage in goats. Workers at Purdue University reported that one passage of scrapie virus in mink rendered it nonpathogenic for mice; if true, this finding may explain the resistance of mice to EN virus.

Studies on the pathogenesis of the Pullman isolate of Aleutian disease in pastel mink are nearly complete. This color phase, in contrast to Sapphire mink, is comparatively resistant. As evidenced by viremia, irrespective of any slight rise in serum gamma globulin, 17 of 20 mink became infected, but only 4 of the 17 developed disease. At 2 years after inoculation, only 1 of 7 infected but not diseased mink had virus persisting in mesenteric node and spleen. Pastels were comparable to Sapphires in their susceptibility to the Utah isolate, which obviously has biological properties different from those of the Pullman isolate. Preliminary results of attempts to cultivate the Utah isolate in tissue culture are encouraging. Virus has now been demonstrated in 4th passage material, but the extent of replication has not been determined. (Hadlow, Jackson, Race)

Serial No. NIAID-170
1. Rocky Mountain Laboratory
2. Office of the Director
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Office of the Director

Previous Serial Number: Same

Principal Investigator: H. G. Stoenner, Director

Other Investigator: R. N. Philip, Assistant Director

Cooperating Units: None

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 41 5/12 | | | 41 5/12 |
| Professional: | 11/12 | | | 11/12 |
| Other: | 40 6/12 | | | 40 6/12 |

Project Description:

The general administration of the laboratory, supervision of research programs, personnel management, budget and fiscal operations, and research support services are combined in this project. During the year, the research sections were reorganized from 9 to 7 sections to improve efficiency, consolidate some program activities, and, in several cases, to foster more effective leadership by section heads.

Honors and Awards:

Dr. H. G. Stoenner

Continued as a Reviewer for American Journal of Veterinary Research.

Continued service on the Committee on Environmental Health of the Montana State Advisory Council on Comprehensive Health Planning.

Continued service on the Board of Directors of the Montana Division of the American Cancer Society.

Co-chairman Leptospirosis Committee of the U.S. Animal Health Assn.

Serial No. NIAID-171
1. Rocky Mountain Laboratory
2. Rickettsial Diseases Sect.
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Rickettsiae and rickettsial infections

Previous Serial Number: Same

Principal Investigators: W. Burgdorfer, R. A. Ormsbee, and R. L. Anacker

Other Investigators: L. P. Brinton, R. K. Gerloff, R. F. Smith, and L. A. Thomas

Cooperating Units: Dr. J. C. Cooney, Tennessee Valley Authority, Muscle Shoals, Alabama
Dr. B. Elisberg, Department of Rickettsial Diseases, WRAIR, Washington, D.C.
Dr. P. Fiset, University of Maryland School of Medicine, Baltimore
Dr. P. A. Gross, VA Hospital, West Haven, Conn.
Dr. Bruce Hudson, CDC, Fort Collins, Colorado
Dr. J. L. Lancaster, Jr., Department of Entomology, University of Arkansas, Fayetteville
Dr. J. J. Plorde, Harborview Medical Center, Seattle, Wn.
Dr. R. Silberman, VA Hospital, Confederate Memorial Medical Center, Shreveport, Louisiana
Dr. E. Weiss, Department of Microbiology, National Naval Medical Center, Bethesda, Md.
WHO, Geneva, Switzerland
Dr. J. D. Aycock and Dr. D. R. Franz, Veterinary Activities, Department of the Army, Fort Hood, Texas

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 14 6/12 | | | 14 6/12 |
| Professional: | 3 6/12 | | | 3 6/12 |
| Other: | 11 | | | 11 |

Project Description:

Objectives:

1) Studies of the ecology and epidemiology of rickettsioses in selected areas of the world,including the United States, 2) development and improvement of serologic and microbiologic methods useful in research of rickettsial diseases, 3) development of improved diagnostic reagents and tests, 4) antigenic analyses of natural groups of pathogenic rickettsiae, i.e. spotted fever group, typhus group, Q fever, 5) study of rickettsial growth and survival under experimental conditions, 6) study of phase change in Coxiella burneti, 7) search for chronic and recrudescent Q fever, and 8) study of immune response in animals and man to rickettsial agents.

Methods employed:

Standard methods applied to rickettsial research in the field and in the laboratory have been employed. In addition, a variety of immunologic, chromatographic and electrophoretic techniques, plus improved modifications of these, have been used in preparation of purified rickettsiae and in studying immune responses.

Major findings:

1. (Burgdorfer) The final results of the ecologic study in "Land Between the Lakes", a 170,000 acre recreational area in the Tennessee Valley Region, showed presence of CF antibodies to the spotted fever group in 8 of 23 species of medium-sized mammals. Fifty-four of 666 sera had CF antibodies to spotted fever antigens, with higher rates among cottontail rabbits (32 of 66), raccoons (9 of 160), and woodchucks (6 of 38). CF antibodies to typhus group rickettsiae in one each of gray fox, raccoon, and feral dog suggested the presence of Rickettsia typhi. The American dog tick, Dermacentor variabilis, was the only species from which R. rickettsi was isolated. Of 931 adults taken from host animals or by dragging, 51 (5.5%) were infected. A rickettsialike organism antigenically related to the spotted fever group but nonpathogenic for laboratory animals was noted in 64 of 545 adult Amblyomma americanum.

A tick survey in the areas surrounding Fayetteville, Arkansas, where several fatal cases of spotted fever have occurred during the past two years, yielded 214 D. variabilis, 482 A. americanum, and 102 Ixodes scapularis. Spotted fever rickettsiae were isolated from 19 (8.8%) D. variabilis, 13 of which came from pet dogs. Rickettsialike organisms that reacted with antispotted fever group conjugates but proved nonpathogenic for laboratory animals were detected in 194 (41.9%) A. americanum; this group included a partially engorged female removed from a patient suffering chills and fever. A rickettsia that reacted against both spotted fever and typhus group conjugates was recorded in 7 (6.8%) I. scapularis. Attempts to isolate in laboratory animals the organisms from both A. americanum and I. scapularis have been uniformly negative.

Isolation of R. rickettsi from 20 of 2,579 immature and adult Haemaphysalis leporispalustris, taken off 133 cottontails (Sylvilagus floridanus) in a highly endemic spotted fever area in Alabama, indicates that this lagomorph may serve as a source of rickettsial infection for ticks. However, the extremely low infection rate (0.7%) among H. leporis-palustris does not explain the high percentage (varying from 50 to 100) of cottontails with CF antibodies to spotted fever group rickettsiae. Since D. variabilis, the well recognized vector of spotted fever in eastern U.S., rarely feeds on cottontails, it appears that the antibodies in a large percentage of rabbits may be nonspecific.

2. (Thomas) In a collaborative study with Dr. Aycock on the incidence of Q fever antibodies in white-tailed deer (Odocoileus virginianus) on the reservation at Fort Hood, Texas, 200 sera were subjected to CF, radioisotope precipitation (RIP), and microagglutination (MA) tests. Although no CF antibodies were detected, 14 sera were positive for MA antibodies against phase II antigen (2 of these also contained phase I antibodies). Of 178 additional sera tested only by CF and RIP tests, 2 were positive by the CF test and 31 by the RIP test.

3. (Ormsbee) The MA test has been shown to be a sensitive and reliable test for the detection of rickettsial antibodies in human sera. In a study in which serologic methods were compared in tests of 1628 sera taken during the past 10 years from RML employees who had been vaccinated with phase I Q fever vaccine, the correlation between results of phase II MA and RIP tests was excellent but that between phase I MA and RIP tests was not at RIP titers of 1:3200 or lower. The CF test was relatively insensitive compared to the MA and RIP tests. In another comparative study involving acute and convalescent sera from 128 Ethiopian patients that were tested against R. prowazeki, R. typhi, R. conori, R. rickettsi, R. akari, and C. burneti, the CF test proved to be the method of choice for specific diagnosis of clinical spotted fever and typhus. In the MA test, extensive cross reactions with spotted fever group antigens were seen with typhus group antibodies and vice versa. Similar cross-reactivity was also seen in MA and immunofluorescence tests of sera of patients in the United States, clinically diagnosed as having either spotted fever or murine typhus.

Much of the serologic data related to the hypothesis of an animal reservoir of epidemic typhus rickettsiae in various parts of Africa was obtained by agglutination tests. Our MA results clearly demonstrated the existence of antigens possessed in common by rickettsiae of the typhus and spotted fever groups. Since spotted fever group infections are widely distributed in African ticks which feed on domestic and wild animals as well as on man, it appears probable that serologic results are due, in fact, to spotted fever group infections that may have been interpreted erroneously to be R. prowazeki infections. A review of early as well as current data leads to the conclusion that considerable antigenic diversity probably exists within the spotted fever-typhus groups, and that it is therefore very difficult to identify the etiologic agent on the basis of agglutination tests alone.

A collaborative investigation with Drs. Weiss, Tyeryar, and Elisberg has shown that, in terms of guanine/cytosine ratios, Rochalimea quintana is only remotely related to Rickettsia; R. akari is clearly distinguishable from R. prowazeki and R. typhi, and R. canada falls between the typhus and and the spotted fever groups but is more closely related to the typhus group.

4. (Ormsbee) Whether C. burneti in phase II will revert, under host pressure, to phase I was examined by first subculturing in embryonated eggs several plaques of a phase II strain (90 egg passages) cultured in a chick embryo cell monolayer. The cloned phase II strain was then passed in guinea pigs and the sera were monitored for phase I antibodies. There was no evidence of reversion to phase I, and it was impossible to transmit the infection through more than two consecutive guinea pig passages. This supports the original hypothesis that phase II C. burneti cannot survive long in mammalian hosts, and that strains of this rickettsia recovered from nature are always in phase I.

A comparison of the 1/M-44 strain of C. burneti, the attenuated phase II strain used in the U.S.S.R. as a live vaccine for humans, with the Nine Mile (90 EP) phase II strain and the Nine Mile (306 GP/2 EP) phase I strain, showed the Russian strain to be quite comparable in pathogenicity to the Nine Mile phase II strain. The 1/M-44 strain was slightly more infectious for mice, and the difference between its $GPID_{50}$ (guinea pig 50% infectious dose) and its $GPFD_{50}$ (guinea pig 50% fever dose) was only 4.6 logs, whereas that for the Nine Mile phase II was 5.5 logs. In contrast, infectious endpoints for Nine Mile phase I in all systems were very close to one rickettsia. In experimentally infected guinea pigs, rickettsiae of both 1/M-44 and cloned Nine Mile phase II strains persisted for at least 30 days but not for 96 days in tissues including spleen, liver, and kidney; recrudescent infection in fetal membranes or placenta was not demonstrated. In guinea pigs neither phase II strain elicited phase I antibodies nor could phase I antibodies be elicited as an anamnestic response in animals challenged with phase I antigen 138 days after infection with either phase II strain. A phase II anamnestic response, however, was readily elicited under these conditions.

Published reports of recrudescent Q fever in fatal cases of rickettsial endocarditis and of massive placental infections in pregnant women prompted us to look for evidence of Q fever infection in patients with chronic pulmonary disease, and in pregnant women. No evidence of recrudescent Q fever was detected during a 3-year study of 40 patients with chronic pulmonary disease. Similarly, we were unable to isolate Q fever rickettsiae from 51 placental specimens tested at random from a group of 142 collected at full term, even though 70% of the women had phase II MA titers from 1:8 to 1:128 at time of delivery.

5. (Anacker) The method for producing purified, particulate rickettsial antigen (see last year's annual report) has so far been applied to 7 species of rickettsiae representing all major groups. These antigens were found stable, did not become anticomplementary for 12 to 18 months, and could be used interchangeably in agglutination and CF tests.

Spotted fever rickettsiae were purified from yolk sacs of embryonated eggs and from mouse L cells by centrifugation in a reorienting density gradient zonal rotor. Vaccine doses of 0.5 to 3.2 µg (dry weight) of such preparations reduced fever in guinea pigs challenged with $10^5$ $ID_{50}$ of R. rickettsi to half that observed in controls. In contrast, 23 µg of commercial vaccine were required to protect guinea pigs to the same degree. One-tenth µg of rickettsiae protected 50% of mice from the lethal effects of 2 $LD_{50}$ of R. rickettsi toxin. Rickettsiae purified by this method fixed complement.

6. (Gerloff) Mass production of spotted fever rickettsiae was obtained in mouse L cell monolayers in Medium 199 with 2% fetal calf serum and 5 units of penicillin per ml. At harvest, infected cells were concentrated, washed in M/15 PBS, pH 7.0, and frozen in sealed tubes as drained packed cell pellets. Yield per gram tissue was at least 10 times greater from these cells than from yolk sacs. Electronmicroscopy revealed rickettsiae with dense protoplasm and large electrontransparent capsules.

Primary cultures of kidney tissue cells from various species of animals (mouse, swine and guinea pig) were equally productive for growing R. sibirica as were mouse L cells and yolk sac tissues. In all three types of kidney cells there was a marked stimulation of acid production by the host cell—a phenomenon not observed in infected L cells. R. canada and C. burneti (phase I and II) grew to nearly the same titers in mouse and swine kidney cultures as in L cells, but lower titers than those in yolk sacs. CPE was present in kidney cells but was different in nature than that in L cells.

7. (Ormsbee) The primary chick embryo plaque assay technique has been used to measure quantitative differences among various species of rickettsiae, with respect to their ability to multiply in eggs after death of the chick embryo. R. rickettsi was found to grow for 72 hours and to increase the antigenic mass by 40-fold. A 75-fold increase was recorded during the same period for R. conori, whereas only a 4.5-fold increase occurred with R. akari. R. prowazeki did not grow after death of the embryo—a characteristic long established for this agent.

Tissue culture systems lend themselves effectively for the assay of antibiotic activity against rickettsiae. This has been shown in a study of sensitivity of R. typhi and R. rickettsi to antibiotics in terms of rickettsial plaque inhibition in primary chick embryo tissue cells. The concentrations of penicillin, streptomycin, and tetracycline which cause 100% inhibition in tissue culture were similar to those which inhibited

rickettsial growth in eggs. Gentamycin inhibited growth in concentrations of about one-quarter those of penicillin and streptomycin. There appeared to be little difference in sensitivity between R. typhi and R. rickettsi to the antibiotics thus far tested.

8. WHO Reference Center function. As a WHO Reference Center, RML last year supplied, on request from 13 domestic and foreign laboratories, 8 antigen preparations for agglutination or CF tests against R. rickettsi, R. prowazeki, and R. typhi, 14 live seeds of C. burneti, R. rickettsi, R. montana, R. conori, R. prowazeki, R. typhi, and Chlamydia psittaci, and 3 antisera against C. burneti.

The first phase of the WHO sponsored collaborative study on laboratory diagnosis of rickettsial infections has been completed. In this phase, RML, along with 6 other rickettsial research laboratories in various parts of the world, provided sera to be tested for CF and/or MA antibodies employing epidemic typhus, endemic typhus, spotted fever, and Q fever antigens prepared in the various participating laboratories. All sera were tested before they were sent along with the results to WHO where they were coded and redistributed to the various laboratories for retesting. In general, there was good agreement among all laboratories, although discrepancies did occur.

9. See Project NIAID-186 for related research on rickettsial agents.

Significance to biomedical research and the program of the Institute:

In view of the steadily increasing number of spotted fever cases in this country it is important to keep health authorities and the general public informed on the prevalence and dynamics of R. rickettsi in nature. This is of particular value for areas, such as "Land Between the Lakes", that are being developed for major outdoor recreation and conservation education. Studies on the ecology of spotted fever rickettsiae have continued to provide interesting information. Detection in A. americanum of a rickettsia related to the spotted fever group but nonpathogenic for laboratory animals, and in I. scapularis of a rickettsialike organism related by immunofluorescence to both spotted fever and typhus groups, suggests the existence in nature of hitherto undescribed rickettsiae that may be pathogenic to man. The role of certain mammals, e.g. cottontails, is far from certain. A high percentage of these animals are seropositive, but they do not appear to be very efficient in transmitting the rickettsia to ticks.

Rickettsial research at RML continues to contribute significantly toward improvement of serologic test procedures and of diagnostic reagents. The MA test has many important advantages over other serologic procedures: its application is relatively simple, does not require elaborate and expensive equipment, standardized reagents or refrigeration, and can be used in any laboratory or even in the field.

The method of purifying rickettsiae without the use of ether has produced antigens from rickettsiae representing all major groups. These preparations have proven remarkably stable and useful in both agglutination and CF tests. A new method, zonal centrifugation, has given preparations of purified R. rickettsi far more potent than had been available so far. This technique is simple and may become the method of choice in preparation of rickettsial antigens for vaccine and diagnostic purposes. Application of mouse L cells to mass production of spotted fever and possibly other rickettsiae is an important step toward preparation of more effective diagnostic reagents.

Knowledge of antigenic diversity in the group of pathogenic Rickettsia is important in interpreting results of epidemiologic and serologic surveys which may have a direct bearing on control measures against rickettsial diseases.

The phase change in C. burneti is associated with other physiologic changes including pathogenicity, virulence, and immunogenicity, and makes the use of live, attenuated vaccines against Q fever possible. The nature, control, and degree of stability of such changes are still not fully explored but must be thoroughly understood if vaccines are to be used safely and effectively.

The nature of the phase I antigenic component of C. burneti makes it different from all other rickettsial antigens. Its apparent role as an adjuvant in immunologic processes and its great chemical stability suggest the possibility of its use in immunization against other diseases, including cancer.

Studies of growth dynamics, DNA characteristics, and antibiotic susceptibility broaden our knowledge of this group of agents and provide a better understanding of the nature and mechanisms of intracellular parasitism.

The WHO supported collaborative study on laboratory diagnosis of rickettsial infections will eventually lead to the long overdue standardization of serologic techniques and their reagents.

Problems of chronic and recrudescent Q fever have been demonstrated in various parts of the world. Their extent and frequency in the U.S., where they undoubtedly occur, must be assessed.

Proposed course of project:

This laboratory will continue to advise and/or assist other institutions in ecologic investigations of spotted fever and other rickettsial diseases in this country. In the study with Dr. J. L. Lancaster, Arkansas, emphasis will be placed on additional field-collected ticks, particularly I. scapularis and A. americanum. In Alabama where the cottontail-H. leporispalustris study nears completion, efforts will be directed toward clarifying the role of small rodents (mice, voles, rats) as reservoirs of

spotted fever and possibly other rickettsiae; this represents the last phase in the cooperative project initiated in 1970 with Dr. J. Cooney, TVA, Muscle Shoals, Alabama.

A cooperative study with Dr. Edward Murray, Harvard School of Public Health, related to the ecology of R. rickettsi on Cape Cod (Massachusetts) is planned but may not materialize because of shortage of funds.

Zonal centrifugation for purification of rickettsiae will be assessed further. Fractionation of purified rickettsiae to isolate and characterize the protective antigen(s) and those indicating phylogenetic relationships will be attempted.

Further studies are planned on immune response of experimental animals to rickettsiae (with emphasis on R. rickettsi and C. burneti) to characterize the types of immunoglobulins evoked. Cellular immune responses and the adjuvant activity of C. burneti in a variety of in vivo and in vitro systems, including experimental tumors, will be investigated. Antigenic variation, particularly phase shift in C. burneti, will receive further attention.

Use of spinner cultures of mouse L cells for mass production of rickettsiae will be explored.

RML will continue to participate in the WHO sponsored world-wide program on laboratory diagnosis of rickettsial infections.

For additional planned studies see NIAID-186.

Honors and Awards:

Dr. Willy Burgdorfer

Continued to serve as Associate Member of the Commission on Rickettsial Diseases, Armed Forces Epidemiological Board.

By invitation participated and presented paper at International Symposium on the Control of Lice and Louse-Borne Diseases, Pan American Health Organization, Washington, D.C., Dec. 4-6, 1972.

Dr. R. A. Ormsbee

Continued to serve as Member of the Commission on Rickettsial Diseases, Armed Forces Epidemiological Board.

By invitation participated and presented paper at International Symposium on the Control of Lice and Louse-Borne Diseases, Pan American Health Organization, Washington, D.C., Dec. 4-6, 1972.

Publications:

Wike, D. A., Tallent, G., Peacock, M. G., and Ormsbee, R. A.: Studies of the rickettsial plaque assay technique. Infect. Immun. 5: 715-722, 1972.

Wike, D. A., Ormsbee, R. A., Tallent, G., and Peacock, M. G.: Effects of various suspending media on plaque formation by rickettsiae in tissue culture. Infect. Immun. 6: 550-556, 1972.

Wike, D. A. and Burgdorfer, W.: Plaque formation in tissue cultures by Rickettsia rickettsi isolated directly from whole blood and tick hemolymph. Infect. Immun. 6: 737-738, 1972.

Burgdorfer, W., Ormsbee, R. A., and Hoogstraal, W.: Ticks as vectors of Rickettsia prowazeki--a controversial issue. Am. J. Trop. Med. Hyg. 21: 989-998, 1972.

Bell, E. J., Munoz, J. J., Peacock, M., and Cole, R. L.: Murine typhus toxin: studies on identification of the neutralizing factor present in normal human serum. Infect. Immun. 6: 232-239, 1972.

In press:

Burgdorfer, W.: Rickettsioses (Introduction, Rocky Mountain spotted fever, Boutonneuse fever, North Asian tick typhus, Queensland tick typhus, rickettsialpox, murine (flea-borne) typhus fever, scrub typhus, Q fever). In Hubbert, W. T., McCulloch, W. F., and Schnurrenberger, P. R. (Eds.): Diseases Transmitted from Animals to Man (ed. 6).

Burgdorfer, W., Ormsbee, R. A., Schmidt, M. L., and Hoogstraal, H.: Search for epidemic typhus agent, Rickettsia prowazeki, in Ethiopian ticks. Bull. WHO

Burgdorfer, W.: Possible extra-human reservoirs of Rickettsia prowazeki: ticks. Pan American Hlth Org. Scientific publications.

Ormsbee, R. A.: The hypothesis of extrahuman reservoirs of Rickettsia prowazeki. Pan American Hlth Org. Scientific publications.

Tyeryar, F. J., Jr., Weiss, E., Miller, D. B., Bozeman, F. M., and Ormsbee, R. A.: DNA base composition of rickettsiae. Science

Serial No. NIAID-173
1. Rocky Mountain Laboratory
2. Zoonoses Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Selected zoonoses of regional importance

Previous Serial Number: Same

Principal Investigator: J. F. Bell

Other Investigators: J. J. Munoz, R. K. Bergman, and H. G. Stoenner

Cooperating Units: Dr. Alvaro Duenas, Universidad del Valle, Cali, Colombia
Dr. Robert Hoffman, Department of Zoology, University of Kansas, Lawrence
Dr. Jack Ward, Veterinary Practitioner, Hamilton, Mont.
Dr. J. E. Catlin, Veterinary Research Laboratory, Montana State University, Bozeman
Dr. Shoichiro Ohara, Director, Laboratory of Ohara General Hospital, Fukushima, Japan
Dr. Nobuo Kusonoki, Dean, Fukushima Medical College
Dr. Yo Yamagata, Assistant Professor, Fukushima Medical College, Japan

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 3 11/12 | | | 3 11/12 |
| Professional: | 1 3/12 | | | 1 3/12 |
| Other: | 2 8/12 | | | 2 8/12 |

Project Description:

Objectives:

Elucidation of the ecology of Francisella tularensis, rabies virus, and other zoonotic microorganisms, and investigation of the pathogenesis and immunology of some zoonoses.

Methods employed:

Field work in ecology and routine laboratory techniques of microbiology, entomology, and immunology are employed as necessary.

Major findings:

1. Epizootiology of tularemia, type B. The less virulent form of F. tularensis (type B), the "aquatic" form, typically infects rodents such as beaver, muskrats and voles. During outbreaks of tularemia in those species, the organism can be isolated from wells, roadside ditches, pools, and clear-running streams, all of which often serve as a source of infection for man. Some waters are continuously contaminated with F. tularensis, and circumstantial evidence suggests infected rodents as the likely source. However, with one exception (few have been tested) observed in our laboratory, rodents in nature are not known to chronically shed the organism. This condition can be produced artificially, but the known methods do not simulate circumstances that would be found in nature. Activities during the past year are listed.

a. An extensive survey in an enzootic area to find voles chronically shedding the organism was defeated by "complete" disappearance of voles from the study area.

b. Chronic tularemia with bacteriuria was produced in large numbers of Microtus pennsylvanicus by vaccination with live F. tularensis of a human vaccine strain (Russian origin) and subsequent inoculation with fully virulent type B organisms. Gross lesions were produced in the kidney - a clear-cut example, it would seem, of nonascending, blood-borne, bacterial nephritis. The histopathology of lesions in kidneys and other organs is being studied by our Japanese collaborators, who state that the lesions are different from any seen previously.

c. Attempts are being made to produce the chronic nephritic form by oral administration of sublethal doses of virulent type B organisms. This method, if successful, could be considered a simulation of natural conditions.

2. Rabies. Studies of the effects of hyperthermia upon mortality and survival after onset in mice infected with low passage rabies virus have been continued. In addition, the effect of hyperthermia on infections with temperature sensitive mutants (from Wistar) is being evaluated. We recently showed that hyperthermia not only resulted in reduction in mortality (roughly proportional to time of exposure at high temperature during the incubation period)but also caused an increase in the rate of abortive infection (a distinct phenomenon). Present efforts are directed toward elucidation of the mechanism by which hyperthermia influences the outcome of the disease.

Continued investigation (with Dr. Munoz) of the enhancing effects of Bordetella pertussis extracts (BPE) upon immunogenesis has shown that the extract, when added to the relatively poor commercial duck embryo vaccine, significantly accelerates development of resistance to challenge. Recent experiments with purified BPE fractions indicate parallelism of that effect with the histamine sensitizing factor. Possible adverse effects of BPE used in vaccine are anergy to vaccine administered after primary injection of the extract and shortening of the incubation period. This project now includes vaccine studies in dogs.

In limited trials, the inoculation of BCG in conjunction with rabies virus reduced mortality and enhanced survival after onset. Those tests, completed in 1961 but not extended because of limited facilities, will be pursued further.

Continued investigations on the immune mechanisms in the central nervous system (CNS) have yielded the following findings.

a. An anomalous significant increase in rabies antibody levels in cerebral spinal fluid occurred in a cat and dogs kept for several years after recovery. The increases were unexpected and remain unexplained. We could not isolate virus from the sputums of the animals, and there was no clinical evidence of awakened infection.

b. There is no indication that experimental allergic encephalomyelitis causes breaching of the blood/brain barrier in cavies, nor have we found that CNS viral infections other than rabies will do so. However, it is becoming apparent that various forms of CNS infection elicit quite different reactions, e.g., equine encephalomyelitis virus infection of the CNS in dogs does not cause development of antibodies in the CNS. It would be desirable to determine the effects of canine distemper encephalitis, especially in view of the bizarre infections of the CNS in man caused by the closely related measles virus. However, distemper virus cannot be used at RML because of the theoretical danger to mink. Hence, we will test the effects of canine herpes virus on the blood/brain barrier.

c. Dr. Alvaro Duenas, Cali, Colombia, will soon publish results of a study of a series of human cases of rabies. He believes there is some indication of chronic or abortive rabies but does not have facilities for serologic tests. We are collaborating with him by testing serums and cerebral spinal fluids.

3. Rabies in bats. Infected bats were found for the 19th consecutive year. Virus was recovered from 2 of 36 (one Lasionycteris and one Myotis) bats submitted. We are continuing surveys (with Dr. Robert Hoffman) of the species distribution of bats in Montana.

4. Application of hypersensitivity therapy of superficial tumors to veterinary practice (cooperation with Dr. Jack Catlin, Montana State University, and Dr. Jack Ward, Veterinary Practitioner). Dr. Edmund Klein (Roswell Park) has achieved spectacular results in treatment of skin carcinoma in man by elicitation of nonspecific hypersensitivity reaction. We are now engaged in testing the applicability of the method to the treatment of superficial tumors in livestock and on superficial tumors induced in laboratory animals (rabbit papilloma). Preliminary tests with sensitizing agents indicate that dinitrochlorobenzene is useful for sensitizing horses and guinea pigs but, under the conditions of trial, not useful for rabbits or cattle. Live F. tularensis (the avirulent human vaccine strain) sensitizes horses and rabbits effectively. To date, F. tularensis has been used to sensitize a horse with sarcoid, in which a reaction will be elicited with F. tularensis cell wall antigen.

5. The biology of Borrelia hermsi in Kelly's culture medium. The study was undertaken to explore the role of antibody in relapsing fever. In the course of infection in rats and mice at least 4 different serotypes have been recognized. It is postulated that relapses occur because the organism can change its surface antigens so that they are not recognized by the antibodies produced in response to preceding relapses. However, it has not been resolved that the different serotypes emerge from a homogeneous population of a single serotype. Conceivably the vector tick contains a mixture of serotypes that appear individually during the usual course of the disease.

Excellent growth has been obtained in 15 passages in Kelly's medium, which is the first medium successfully used for growing relapsing fever spirochetes. A method has been devised for initiating growth from a single organism. Progress in the project was impeded by our inability to produce serotype-specific antiserum in rabbits by published procedures. Recently, some serotype-specific antiserums were obtained from mice bled within 5 days after inoculation with organisms isolated from four successive relapses in a single mouse. With these reagents we hope to identify changes in serotypes that may occur in response to antibody pressure in culture medium.

Significance to biomedical research and the program of the Institute:

Present research is designed to fill the one hiatus remaining in our concept of the source of F. tularensis type B in streams. We know that chronic shedder state can occur in voles in nature and can be produced in voles experimentally in the laboratory by an artificial method. We do not know how chronic infection can be produced in nature but hope that current experiments will provide a clue.

There is currently a consensus that bacterial nephritis is a result of ascending infection, but tularemia nephritis, as we produce it, results from organisms introduced parenterally, and it is much more likely a result of bacteremia. Currently, the nephritic kidneys are being studied to identify possible differential histopathologic characteristics between this form of nephritis and that resulting from ascending infection. So far, the lesions seen are different from those in other forms of tularemia infection.

Previously we had concluded that hyperthermia in mice induced by elevated ambient temperature diminished mortality from rabies only because of decreased established infection. Recently we have found that, under certain conditions, established infections are aborted more frequently at high than at lower ambient temperature. Applicability of these findings to medical or veterinary practice, epidemiology, and therapy must await elucidation of the mechanisms.

Treatment of rabies-exposed patients typically is a matter of urgency as well as apprehension and difficult decision. When a decision is made to treat, ordinarily some time has elapsed since exposure. Vaccines in current use in the United States are notoriously weakly antigenic, and in the race between development of disease and development of immunity the latter is handicapped. The WHO Expert Committee recommends administration of immune globulin for immediate treatment (in addition to vaccine) but this treatment has several disadvantages: 1) immune globulin inhibits development of active immunity and necessitates additional vaccination, 2) heterologous globulin now used causes frequent severe allergic reactions, 3) homologous globulin is much more expensive and has very limited distribution, 4) evidence that immune globulin is really effective under practical circumstances is limited and even controversial, and 5) throughout the world it is available to perhaps one in 100 persons exposed. Therefore, the use of BPE to accelerate and augment the immune response to vaccine may be more effective, less dangerous, and much more generally available.

Evidence that only some kinds of encephalitis result in antibody (formation) in the CNS has posed a problem of identifying the conditions that control this phenomenon. Also, in the past year it has been found at CDC that abortion of infection can occur at the level of the ganglion (with monoplegia), and in that event CNS antibody levels are not elevated. According to some, the blood/brain barrier extends to the periphery of nerve distribution. The latter concept would seem to be incompatible with low CNS antibody levels in ganglion-limited infection. Those questions and the observation that CNS antibody levels can rise years after clinical illness are phenomena of practical importance in pathogenesis and immunity.

The inquiry into hypersensitivity therapy for superficial tumors in domestic animals has the practical objective of finding a cure for economically important diseases of livestock and pets (e.g., cancer eye in

cattle, sarcoid in horses). It also affords nearly unlimited opportunity for evaluation of this type of therapy in species much more subject than man to manipulation.

Proposed course of project:

We hope to conclude studies on the ecology of F. tularensis type B this year unless student help becomes available to investigate our earlier finding of the organism in soil. Inquiry into the pathogenesis and pathology of tularemia nephritis will be continued in collaboration with Japanese collaborators.

Studies of CNS immunity are being broadened to include infectious encephalitis other than rabies in an attempt to characterize those that do, and do not, elicit CNS antibody. We will continue to investigate the conditions that are conducive to abortion of infection. The finding that hyperthermia tends to abort active infection, as well as inhibit establishment of infection, encourages further study which will be continued.

The effects of B. pertussis extract upon the immune response to live virus and various vaccines will be studied further. Relatively pure fractions are now being used, in parallel with other studies of those fractions (by Dr. Munoz).

We will continue surveillance of diseases of various wild and domestic animals, as in previous years, as a service to state and county public health agencies and veterinary and medical practitioners. Diagnostic service for suspected rabies in bats and other animals will be continued.

Honors and Awards:

Dr. J. F. Bell

Appointed to Governor's (Montana) Interagency Council on Rodent, Predator and Rabies Control.

Reappointed Adjunct Professor, Zoology Department, University of Montana.

Publications:

Delpietro, H., Diaz, A.M.C., Fuenzalida, E., and Bell, J. F.: Determinacion de la tasa de ataque de rabia en murcielagos. Bol. Ofic. Sanit. Panamer. 73: 222-230, 1972.

In press:

Bell, J. F. and Munoz, J. J.: Enhancement of immune response to rabies virus by Bordetella pertussis extract. Proc. Symposium sur la Rage, Lyon, France, 1972.

Bell, J. F.: Latency - abortive rabies. In Baer, G. M. (Ed.): The Natural History of Rabies. New York, Academic Press.

Serial No. NIAID-174
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Systematics and vector relationships of certain parasitic arthropods

Previous Serial Number: Same

Principal Investigator: J. M. Brennan

Other Investigators: C. M. Clifford, J. E. Keirans, and C. E. Yunker

Cooperating Units:
Dr. J.E.M.H. van Bronswijk, Academisch Ziekunhuis Utrecht, Utrecht, The Netherlands
Dr. M. Chitwood, National Center for Primate Biology, Davis, Calif.
Dr. A. Fain, Institute of Tropical Medicine, Antwerp, Belgium
Dr. C. O. Handley, Smithsonian Institution
Dr. S. Herrin, Univ. of Southern Georgia, Statesboro
Dr. H. Hoogstraal, NAMRU-3, Cairo, Egypt
Dr. K. Johnson, MARU, Canal Zone
Dr. J. Jones, Texas Technical University, Lubbock
Dr. G. W. Krantz, Oregon State University, Corvallis
Dr. R. B. Loomis, California State Univ., Long Beach
Dr. F. Lukoschus, Katholieke Universiteit, Nijmegen, The Netherlands
Dr. C. MacFarland, University of Wisconsin, Madison
Dr. R. Parker, Sorvall Inc., Hamilton
Dr. F. J. Radovsky, Bernice P. Bishop Museum, Honolulu, Hawaii
Mr. J. T. Reed, Brigham Young University, Provo, Utah
Mr. R. Saunders, Brigham Young University, Provo, Utah
Dr. D. Sonenshine, Old Dominion University, Norfolk, Virginia
Dr. V. J. Tipton, Brigham Young University, Provo, Utah

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 6 11/12 | | 1/12 | 7 |
| Professional: | 2 8/12 | | 1/12 | 2 9/12 |
| Other: | 4 3/12 | | | 4 3/12 |

Contract Support:

| Contract Number | Project Officer (NIAID) | Contractor | Project Director | Annual Funding Level |
|---|---|---|---|---|
| PL-480 03-005-1 | C. M. Clifford<br>J. E. Keirans<br>H. G. Stoenner | NAMRU-3<br>Cairo, Egypt | H. Hoogstraal | $53,439 |

Project Description:

Objectives:

Taxonomy and colonization of parasitic arthropods of known and potential medical importance.

Methods employed:

Standard, adapted, and conceived techniques for collecting, handling, rearing and colonizing parasitic arthropods and their preparation for taxonomic study. Collaboration with appropriate sections at RML and other agencies and institutions. Identification and clarification of systematic problems, principally of ticks and parasitic mites.

Major findings:

1. Ticks (Clifford and Keirans). The Bishopp tick collection transferred to the Rocky Mountain Laboratory in 1971 is being integrated into the RML collection. Over 2,900 lots containing many thousands of ticks have been identified. Data from these extensive collections are being programmed on punch tape for input into the Smithsonian data retrieval system.

Approximately 400 collections of ticks from Nepal have been identified and a manuscript describing all known and new species of Ixodes is in preparation.

A collaborative project with personnel at NAMRU-3 and the University of Wisconsin on ticks of the Galápagos Islands is continuing.

Transfer of Dr. Hoogstraal's tick collection and library continued during the year. PL-480 project 03-005-1 included an on-site visit to NAMRU-3 by Drs. Clifford and Yunker, and Mrs. Jones.

2. Biochemical taxonomy of ticks, with special reference to the Haemaphysalis longicornis complex (Yunker and Keirans). Representatives of isolated demes of H. longicornis were collected on various South Pacific islands. These populations, all parthenogenetic, have been established as laboratory colonies, but work with them has been prevented by the loss of our insectary attendant. In addition, funds for collecting nonparthenogenetic H. longicornis and sibling species were cut off late last year and it has not been possible to obtain the cooperation of collectors overseas. The significance to medical research of this project stems from the ability of members of the taxonomically difficult H. longicornis complex to transmit to man or domestic animals a number of viral, rickettsial, or protozoan agents of disease. Regrettably, until insectary capabilities can be restored, this promising study will have to be tabled. Collaborators: Hoogstraal, Parker.

3. Scanning electron microscopy (Yunker, Clifford, and Keirans). A preliminary investigation of the application of scanning electron microscopy (SEM) to the study of medically important Acarina was made. Adult specimens of 4 species of closely related Ornithodoros ticks (capensis group) which are distinguishable only as larvae, were number-coded, divested of other identification and photographed by SEM. Analysis of resulting photos revealed morphological differences and clearly demonstrated that, by SEM, adults are quickly and easily separable. Further studies will be directed toward establishing limits of individual variation within a species.

4. Parasitic mites (Yunker). Identifications of nearly 2,000 collections are complete. One manuscript has been published and 5 are in press. Others are in various stages of completion. Collaborators: Tipton, Saunders, Herrin, Radovsky. A new species of Neotropical entonyssid mite was described.

5. Chiggers (Brennan). Identifications of the Venezuelan collection of some 20,000 specimens were completed. Approximately 150 species, nearly half of them new, are recognized. A rather sizeable report on all of these is being developed and should be in press by the year's end. In the interim, smaller reports on appropriate taxa have been published or

are in preparation. A comprehensive study of the medically important genus Eutrombicula of the Neotropical region is progressing slowly as is a continuing study of Surinam chiggers. Pertinent to this work, 5 manuscripts have been published and 2 are in press. Collaborators: Loomis, Reed, Tipton, van Bronswijk, Lukoschus.

Three new genera and 9 new species of Neotropical chiggers were described, and identifications were made for various organizations and individuals as requested.

A guest worker, Dr. J.E.M.H. van Bronswijk, Utrecht, the Netherlands, spent about 6 weeks in this unit for training in colonizing techniques and systematics of chiggers.

6. Tick, mite, and mosquito colonies. In 1972 the RML insectary provided 155,890 ticks of 8 species for research needs at RML and other institutions. Because we are not authorized to replace our insectary technician, we will no longer be able to supply parasitic arthropods to institutions and investigators throughout the world.

Colonies of the Haemaphysalis longicornis complex have been established from Australia, New Zealand (2 localities), New Hebrides, Fiji, and Tonga to provide material for a project on biochemical taxonomy of ticks in collaboration with Drs. Yunker, Parker, Hoogstraal, and Keirans.

Currently, 29 tick, 3 mite, and 1 mosquito species are under colonization.

Significance to biomedical research and the program of the Institute:

As in the past, where arthropods are concerned in human disease, their systematic studies are basic and a must to all research issuing therefrom. The taxonomically and biologically trained personnel of this section, in addition to providing numerous identifications, are frequently consulted for information involving protection from and control of medically important arthropods, and are even appealed to for aid in solving problems of entomophobia. Over and above basic research, various services are provided. The arthropod colonies are used in arthropod tissue culture, transmission studies, establishing subcolonies, teaching rearing techniques, etc.

Proposed course of project:

Continued investigations of parasitic arthropod systematics and biology, and provision of services, with emphasis on ticks, mites, and mosquitoes.

Publications:

Brennan, J. M. and Reed, J. T.: Two new Venezuelan chiggers of the genus Polylopadium (Acarina: Trombiculidae). J. Med. Entomol. 9: 461-463, 1972.

Keirans, J. E.: A note on the generic names Otobius and Otophilus (Acarina: Argasidae). J. Med. Entomol. 9: 478, 1972.

Fain, A. and Yunker, C. E.: A new species of Pneumophionyssus from Argentina and new locality records of acarine parasites of reptiles in Mexico and Venezuela (Mesostigmata: Entonyssidae and Laelapidae). J. Med. Entomol. 9: 482-484, 1972.

Brennan, J. M. and Reed, J. T.: Loomisia gen. n. with descriptions of three new Venezuelan species (Acarina: Trombiculidae). J. Parasitol. 58: 796-800, 1972.

Goff, M. L., Loomis, R. B., and Brennan, J. M.: Odontacarus dentatus (Ewing, 1925) is the senior synonym of O. galli (Ewing, 1946) (Acarina: Trombiculidae). J. Med. Entomol. 9: 479-481, 1972.

Brennan, J. M.: Buclypeus, new genus for Trombicula ignota Brennan, T. rotundiscutata Fauran, and two new species (Acarina: Trombiculidae). J. Parasitol. 58: 1178-1181, 1972.

Brennan, J. M.: Revival of Vanidicus Brennan & Jones, 1961, descriptions of two new species of the genus from Venezuela, and notes on related taxa (Acarina: Trombiculidae). J. Med. Entomol. 10: 107-109, 1973.

Jones, E. K. and Clifford, C. M.: The systematics of the subfamily Ornithodorinae (Acarina: Argasidae). V. A revised key to larval Argasidae of the Western Hemisphere and description of seven new species of Ornithodoros. Ann. Entomol. Soc. Am. 65: 730-740, 1972.

Keirans, J. E. and Jones, E. K.: Description of the immature stages of Ixodes (I.) sinaloa Kohls and Clifford (Acarina: Ixodidae), from rodents in Mexico and Nicaragua. Acarologia 13: 471-475, 1972.

Keirans, J. E., Backhurst, G. C., and Clifford, C. M.: Description of the male of Ixodes walkerae with notes on the female and description of the males of Ixodes daveyi and Ixodes thomasae (Acarina: Ixodidae). Rev. Zool. Bot. Afr. 85: 59-66, 1972.

Keirans, J. E.: Redescription of Amblyomma fulvum Neumann 1899 (Acarina: Ixodidae), a parasite of the giant anaconda in northern South America. J. Med. Entomol. 9: 138-139, 1972.

Jones, E. K., Clifford, C. M., Keirans, J. E., and Kohls, G. M.: The ticks of Venezuela (Acarina: Ixodoidea) with a key to the species of Amblyomma in the Western Hemisphere. Brigham Young Univ. Sci. Bull. Biological Series 17: 1-40, 1972.

In press:

Brennan, J. M. and Reed, J. T.: More new genera and species of chiggers from Venezuela (Acarina: Trombiculidae). J. Parasitol.

Brennan, J. M. and van Bronswijk, J.E.M.H.: Endoparasitic chiggers. VII. A redescription of Kymocta Yunker and Brennan, 1962, a new species from Venezuela, and other records (Acarina: Trombiculidae). J. Med. Entomol.

Herrin, C. S. and Yunker, C. E.: A new species of Hirstionyssus from Liomys irroratus in Texas (Acari: Mesostigmata). J. Med. Entomol.

Yunker, C. E. and Chitwood, M. B.: Note on the occurrence of larval filariae in a mite parasitic on bats. Acarologia

Yunker, C. E.: Acarina other than ticks. In Flynn, R. J. (Ed.): Diseases of Laboratory Animals (Chapter 15). Iowa State Univ. Press, Ames, Iowa.

Yunker, C. E. and Saunders, R. C.: Venezuelan Macronyssidae. 1. The genera Acanthonyssus Yunker and Radovsky, and Argitis, n. gen. J. Med. Entomol.

Saunders, R. and Yunker, C. E.: Venezuelan Macronyssidae. 2. Nycteronyssus desmodus, n. gen., n. sp., off a vampire bat. J. Med. Entomol.

Stark, H. E., Inlao, I., Clifford, C. M., Keirans, J. E., and Lakshana, P.: A preliminary checklist of the ticks of Thailand. Appl. Sci. Res. Corp. J. Thailand

Clifford, C. M., Sonenshine, D. E., Keirans, J. E., and Kohls, G. M.: The systematics of the subfamily Ixodinae (Acarina: Ixodidae). I. The subgenera of Ixodes. Ann. Entomol. Soc. Am.

Keirans, J. E., Hoogstraal, H., and Clifford, C. M.: The Amblyomma (Acarina: Ixodidae) parasitic on giant tortoises (Reptilia: Testudinidae) of the Galapágos Islands. Ann. Entomol. Soc. Am.

Hoogstraal, H., Clifford, C. M., Saito, Y., and Keirans, J. E.: Ixodes (Partipalpiger) ovatus Neumann, subgen. nov.: identity, hosts, ecology, and distribution (Ixodoidea: Ixodidae). J. Med. Entomol.

Keirans, J. E.: Ixodes (I.) montoyanus Cooley (Acarina: Ixodidae): description of the male and immature stages from dwarf deer in Colombia. J. Med. Entomol.

Hoogstraal, H., Clifford, C. M., and Keirans, J. E.: Argas (Microargas) transversus (Ixodoidea: Argasidae) of Galápagos giant tortoises: description of the female and nymph. Ann. Entomol. Soc. Am.

Serial No. NIAID-175
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Allergy, its mechanisms and role in disease

Previous Serial Number: Same

Principal Investigators: J. J. Munoz, B. W. Chesebro, D. L. Lodmell, J. E. Coe, and R. K. Bergman

Other Investigators: M. E. Bloom, W. J. Hadlow, T. A. Jackson, J. L. Portis, R. E. Race, and L. A. Thomas

Cooperating Units: Dr. A. L. Notkins, Chief, Virology Section, LMI, NIDR
Drs. C. J. Peters and K. M. Johnson, Gorgas Memorial Hospital, Canal Zone, Panama
Dr. Jack Stimpfling, McLaughlin Research Institute, Great Falls, Montana

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 12 4/12 | | 7/12 | 12 11/12 |
| Professional: | 4 9/12 | | 7/12 | 5 4/12 |
| Other: | 7 7/12 | | | 7 7/12 |

Project Description:

Objectives:

1) To study the fundamental bases of hypersensitivity and its role in disease, 2) to learn more about the function of immunoglobulin classes and the factors that affect their production, 3) to investigate the mechanisms of infection in slow virus infections, 4) to study some aspects of tumor immunity, and 5) to study methods of demonstrating viruses that cause chronic infections and to develop immunological methods of diagnosing the disease.

Methods employed:

Typical immunological, biochemical, biophysical, and serological techniques are used. Peroxidase, fluorescent, and isotope labelling of antibodies is employed. Electron microscopy, radioimmunoelectrophoresis and tissue culture techniques are widely used. Rabbits, hamsters, inbred mice, sheep, goats, mink, and guinea pigs are employed in various aspects of this project.

Major findings:

1. Friend virus leukemia (Chesebro). Tumor immunity in the Friend virus leukemia (FVL) system in mice is being studied with particular reference to the relationships between the immune response to tumor-specific cell-surface antigens and the high incidence of recovery from FVL in certain strains of mice whose susceptibility is thought to be controlled by a genetic trait closely linked to the major histocompatibility complex (H-2). Leukemic spleen cells from H-2 (d/d) mice, which rarely recover from FVL, have serologically detectable tumor antigens which decrease markedly during the course of the disease. Similar antigens on leukemic spleen cells from H-2 (b/b) mice, which have a high incidence of recovery from FVL, do not decrease during the course of the disease. Whether these differences in amounts of tumor antigen could account for the difference in incidence of recovery from FVL is being investigated. In addition, 4 transplantable tumor lines have been derived from primary FVL spleen cells. None of these lines appears to have sufficient tumor antigen on their surfaces to be detectable by cytotoxic antibodies; however, all the lines continue to produce low amounts of virus.

An in vitro cell-mediated cytotoxicity assay for FVL tumor antigens has been developed. Primary FVL spleen cells and FV-induced tumor line cells are being compared both in this assay and in vivo for their ability to produce FVL specific tumor immunity. Mice of high (H-2(b/b)) and low (H-2(d/d)) recovery strains are being compared in this cell-mediated cytotoxicity assay to study the role of cellular immunity in the observed differences in recovery from FVL.

Breeding experiments are under way in collaboration with Dr. Jack Stimpfling of the McLaughlin Research Institute, Great Falls, Montana, using mice with proven genetic recombinations within the H-2 complex to determine which area of the complex is responsible for the high incidence of recovery from FVL seen in H-2(b/b) mice.

2. Studies on Aleutian disease (AD) (Chesebro and Bloom). Immunoglobulins from mink 3 to 4 months post-infection with AD have been covalently linked to Sepharose in an attempt to isolate AD virus by immunoabsorption techniques. In addition, isolation of the virus using

conventional virological techniques such as CsCl and sucrose gradient ultracentrifugation is being attempted. Presence of virus is being followed by titration in mink and by electron microscopy and counter immunoelectrophoresis.

3. Studies on scrapie virus (Chesebro). Mice and rabbits have been inoculated with scrapie infectious material complexed to a good immunogenic carrier molecule (methylated bovine serum albumin) in an effort to produce neutralizing or binding antibodies to the scrapie agent. Screening is being done for both binding antibodies and possible scrapie infectious immune complexes with the use of anti-immunoglobulin sera from other species.

4. Immunological tests for slow virus.

a. Complement fixation for AD (Munoz, Hadlow, and Kennedy). The CF test has been reported to be useful to detect AD infection in mink. We obtained some promising results with our antigen preparations and mink sera, but most mink sera from animals affected with AD were highly anti-complementary. Some sera were anticomplementary in dilutions as high as 1/50,000 and most at a dilution of 1/100. We were not totally successful in eliminating this anticomplementary activity (high speed centrifugation, absorption with sheep cells and guinea pig complement, sucrose gradient centrifugation). This problem should be more thoroughly investigated.

b. Delayed hypersensitivity in AD and normal mink (Munoz, Hadlow, and Race). Mink develop delayed hypersensitivity (DH) to PPD when given BCG cell walls in oil-in-water emulsions. The best response was obtained 13-20 days after sensitization, but reactions observed were much weaker than those in guinea pigs. The sapphire mink gave consistently stronger reactions than did pastel mink. AD affected sapphire mink also developed DH to PPD when immunized with BCG cell walls.

c. Peroxidase conjugated antibodies (Bloom). Conjugates have been made of horseradish peroxidase and rabbit antibodies to gamma globulin of mink, goat and guinea pigs. With these reagents we should be able to demonstrate specific binding of globulin to virus or virus containing cells in various systems. It has been possible, so far, to apply this method to demonstrate rickettsiae in infected mouse tissues, and preliminary attempts to demonstrate chronic pneumonitis virus grown in tissue culture are encouraging.

d. Electron microscopy (Bloom and Chesebro). Preparations for electron microscopy of tissues and various fractions obtained from sera of AD affected mink have been made and are presently under study.

5. The roles of humoral and cell-mediated immunity in virus infections (Lodmell). In vitro assays have been used for measuring both humoral and cell-mediated immunity to herpes simplex virus infections in mice. A marked correlation between cytolytic and neutralizing antibody titers has been shown in the humoral immune response, and the immunological destruction of herpes virus-infected cells by immune lymphoid cells has been demonstrated. The role of blocking antibody in the cell-mediated immune response is under investigation.

6. The immunological destruction of virus-infected cells by antiviral Ig and complement (Lodmell). Several viruses, including progressive pneumonia virus of sheep and Aleutian disease virus of mink, are being studied for the possibility that specific antiviral Ig and complement can destroy virus-infected cells and thereby lend insight into the pathogenesis of these diseases.

7. The macrophage and virus-infected cells (Lodmell). An in vitro assay has been developed for determining the effects of macrophages on virus-infected cells. Macrophages were shown to prevent plaque formation and to inhibit viral replication in virus-infected monolayers. It also has been shown that a combination of macrophages, antiviral Ig and complement, can "cure" a virus-infected monolayer. Additional studies on the effect of normal versus specifically "activated" macrophages on virus-infected monolayers are in progress.

8. Hamster serum Ig: Site of synthesis and ontogeny (Coe). Since the 16-day gestation period of the Syrian hamster is one of the shortest of laboratory animals, the neonatal development of the Ig system is of interest. Accordingly, serum concentrations of Ig ($\gamma 2$, $\gamma 1$, $\gamma A$, $\gamma M$) were determined in RML Syrian hamsters at various intervals after birth. Only $7S\gamma 2$ globulin was detected consistently on the day of birth (ave. 0.6 mg/ml), but $\gamma 1$ was occasionally found (ave. 0.05 mg/ml). Serum $7S\gamma 2$ globulin rapidly increased to $\sim$2.5 mg/ml by day 7, although it then decreased by day 21 to 0.75 mg/ml before progressing to adult levels (6-10 mg/ml), which were realized by the 42nd day. $7S\gamma 1$ globulin remained between 0.2-0.5 mg/ml until 42 days after birth and adult levels (2.4-3.0 mg/ml) were not achieved until 105 days of age. Transfer of $7S\gamma 1$ and $7S\gamma 2$ via the milk was indicated, because autoradiographic analysis of neonatal tissues (tested for in vitro incorporation of $C^{14}$ amino acids into Ig) revealed that $\gamma 2$ and $\gamma 1$ syntheses were not detectable until 10 and 20 days, respectively, post partum. $\gamma 1$ synthesis occurred only in lymph nodes, whereas $\gamma 2$ synthesis was also found in spleen tissue. Serum IgM was detected initially 5 days after birth, increased rapidly to $\sim$1.8 mg/ml by day 20, and then declined to adult levels of 0.8-1.0 mg/ml. Synthesis of IgM also was found initially on day 5 in splenic tissue. Synthesis of $\gamma A$ was not detected until day 8 after birth; serum IgA was found 1 day later (day 9) and gradually increased to adult levels (0.2-0.3 mg/ml). Thus, hamster $7S\gamma 1$ appears to be the slowest developing Ig and suggests its relatively recent evolution in the

rodent Ig system. On the other hand, the greatest relative increase in serum concentration after antigenic challenge occurs in the $7S\gamma 1$ class. At 50 days after inoculation with 5 μgm of hen egg albumin in complete Freund's (HEA-CF), $7S\gamma 1$ increased 350% (5.3 mg/ml) whereas $\gamma A$ increased 130%, $7S\gamma 2$ increased 105%, $\gamma M$ increased 65%. Therefore, from the quantitative view, $7S\gamma 1$ appears to have the major role in immune responsiveness. We have previously defined situations in which the antibody response is restricted only to the $7S\gamma 1$ globulins.

Preliminary observations with inbred hamsters indicate that serum Ig concentrations vary according to the strain of hamster. For example, normal LSH hamsters have higher levels of $\gamma A$ ($\sim$3.0 mg/ml) and $\gamma 1$ ($\sim$4 mg/ml) than LHC hamsters ($\gamma A$ = 0.5, $\gamma 1$ = 1.5).

9. Hamster female protein (Coe). A serum protein unique for the adult female hamster has been identified. This electrophoretically fast (alpha) protein, which has a sedimentation value between 6-7S, is initially detectable in the serum of both sexes 10 days after birth, but persists only in adult females. In the male, the protein concentration peaks at day 21 (weaning) and becomes undetectable by day 50; because this female protein was not detected in hamster milk, its transient appearance is most likely due to synthesis in the male. In the female, the protein rapidly increases to adult levels by 4 weeks of age. The transient appearance of this protein in male hamsters apparently renders them tolerant, because adult males do not respond immunologically to this female protein. An antigenically related female protein could not be detected in other rodent species - even in close relatives like the Chinese hamsters. Further research will define the sex-linked factors involved in the synthesis of this protein and its functional role.

10. Other immunological studies on the hamster (Portis and Coe).

a. The hamster cheek pouch is unique in its ability to accept heterologous skin grafts and tumor implants. The reason for this is not clear, but could be due to a deficient response to antigens given by this route. Hamsters receiving HEA by the cheek pouch route responded at a slower rate than those receiving the same doses of HEA IP or by the footpad route. The rate of egress of HEA $I^{131}$ from cheek pouch and footpad was not significantly different.

b. Hamsters immunized with HEA in saline are known to produce mainly $7S\gamma 1$ antibody while those immunized with HEA in complete Freund's adjuvant (CFA) respond with antibody of $7S\gamma 1$ and $7S\gamma 2$ types. Hamsters previously immunized with HEA in saline eliminated approximately 40% of HEA $I^{131}$ within 1 hour after administration. These animals withstood the IV dose of HEA $I^{131}$ without any symptoms. Animals that had been immunized with HEA-CFA eliminated the same dose of HEA $I^{131}$ much slower, since it took 48 hours to eliminate 50% of the HEA $I^{131}$. Most of the animals in this group, however, died within 2-4 hours after antigen administration. The sequestering of antigen in lungs and liver and an acute pulmonary

vasculitis observed suggest an immune complex disease as the cause of death. Animals lacking $7S\gamma 2$ (HEA saline group) did not show this apparent immune complex disease.

c. Hamster peritoneal mast cells, incubated with serum from hamsters that had been infected with Trichinella spiralis 33 days earlier, released 60% of their histamine content when incubated with rabbit anti-hamster $\gamma 1\gamma 2$ serum containing antilight chain antibody. By analogy to other animal systems this observation may indicate the presence of a still undescribed $\gamma E$ immunoglobulin in the hamster.

11. Studies on snake antibodies (Coe and Thomas). Investigations on the role of snakes as vectors of WEE have been difficult because it has been impossible to reliably detect an antibody response to this virus. Preliminary results with an indirect neutralization test ("snake anti-WEE" + WEE + rabbit antisnake serum) indicate that immune snake serum does indeed bind in a specific fashion to WEE. To prepare an antiserum specific for snake Ig, the snake Ig were defined by radioimmunoelectrophoretic examination of sera from snakes immunized with purified protein antigens. Antibodies to human gamma globulin, HEA, and keyhole limpet hemocyanin were detected in most snakes by 48 days postinjection, and specific antigen binding activity was found in 3 separate proteins. Further work is required to characterize the Ig in this surprisingly complex snake Ig system, and to define their role in the anti-WEE response.

12. Studies on Calomys callosus (Coe). We have previously described the $7S\gamma 1$, $7S\gamma 2$, and $\gamma M$ globulins of C. callosus - a natural Machupo virus reservoir. Specific antisera have been made and a Mancini type ring diffusion assay developed for analysis of serum Ig concentration in Machupo infected Calomys. In addition, antisera have been labelled with fluorescein isothiocyanate, so that the presence of Ig complexes in kidneys may be surveyed by direct FA technique, and the Ig class of anti-Machupo virus may be assayed by indirect FA procedures. These studies are presently under way at MARU.

Significance to biomedical research and the program of the Institute:

As indicated in the 1971-72 report, we are presently emphasizing immunological studies with chronic virus diseases and other viruses that can be more conveniently used as models to study various immunological phenomena associated with virus infections. Dr. Chesebro is studying the role of genetic factors and virus induced tumor antigens on cell surfaces on development of Friend virus leukemia of mice. The elucidation of these phenomena would increase our understanding of resistance and susceptibility to leukemic tumors. His research on developing methods of purifying and demonstrating the agents of some chronic viral diseases (AD and scrapie) may provide ways of accelerating studies of these agents.

Dr. Lodmell is studying various in vitro aspects of viral infection and immunity with the aim of understanding the effects of specific and nonspecific factors involved in viral immunity at the cellular level. Since these factors are of obvious importance in recovery from infection, further knowledge along these lines would help understand chronic virus infections.

Dr. Coe's contributions may provide a better understanding of the production and function of various immunoglobulins in a variety of animal species. The hamster has been extensively studied. Since this animal is rather unique in accepting tumors and skin grafts and in showing a marked resistance to anaphylaxis and other hypersensitivity reactions, a thorough study of the immunological mechanism in this animal may provide a better understanding of susceptibility and immunity to tumors and to diseases involving hypersensitivity. The studies on immunoglobulins in cold-blooded animals has shown that these animals may be useful in preparing antibodies to certain antigens which are poorly antigenic in mammals.

Proposed course of project:

The work on chronic virus diseases and on in vitro models to study various immunological aspects of viruses will be continued and intensified. In addition, the studies on production, metabolism and function of various immunoglobulins will be continued.

Honors and Awards:

Dr. J. J. Munoz

Courtesy appointment as Faculty Affiliate, University of Montana.

Served as member of the Council for the Mid-Winter Immunologist Conference.

On Editorial Board of Infection and Immunity.

Invited member of Reticuloendothelial Society.

Dr. J. E. Coe

Courtesy appointment as Faculty Affiliate, University of Montana.

Dr. B. W. Chesebro

Invited speaker at the 3rd International Convocation on Immunology at Buffalo, N.Y., June 1972.

Invited speaker at U.S.-Japanese Cultural Exchange Conference on Immunochemistry and Cellular Immunology, Honolulu, Hawaii, February 1973.

Dr. D. L. Lodmell

Invited to participate in a workshop on "Immunopathology of Virus Infections" held at the Annual Meeting of American Association of Immunologists held in Atlantic City, April 1973.

Publications:

Coe, J. E.: A genetic polymorphism of Peromyscus 7S$\gamma$1 globulins: detection by differences in electrophoretic mobility. J. Immunol. 108: 907-912, 1972.

Coe, J. E.: Immune response in the turtle (Chrysemys picta). Immunology 23: 45-52, 1972.

Coe, J. E. and Takemoto, K. K.: Immune response in the hamster. VI. Antibody response in polyoma oncogenesis. J. Natl. Cancer Inst. 49: 39-44, 1972.

Hayashi, K., Lodmell, D., Rosenthal, J., and Notkins, A. L.: Binding of $^{125}$I-labeled anti-IgG, rheumatoid factor and anti-$C_3$ to immune complexes on the surface of virus-infected cells. J. Immunol. 110: 316-319, 1973.

Lodmell, D. L., Niwa, A., Hayashi, K., and Notkins, A. L.: Prevention of cell-to-cell spread of herpes simplex virus by leukocytes. J. Exp. Med. 137: 706-720, 1973.

Gander, J. E. and Rudbach, J. A.: Immunological investigations of penicillium—II. Primary binding of glycopeptides and glycopeptide derivatives to specific antibodies. Immunochemistry 10: 81-92, 1973.

In press:

Wohlenberg, C. R., Lodmell, D. L., Brier, A. M., and Notkins, A. L.: A quantitative semi-micro assay for the measurement of antiviral antibody cytolytic to cells infected with herpes simplex virus. Appl. Microbiol.

Serial No. NIAID-177
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immune prophylaxis of mycobacterial infections

Previous Serial Number: Same

Principal Investigator: T. J. Meyer

Other Investigators: I. Azuma (NIH Visiting Scientist) and E. Ribi

Cooperating Units: Dr. W. R. Barclay, AMA, Chicago
Dr. W. Brehmer, Robert Koch Institute, Berlin, Germany
Dr. R. Good, Hazleton Laboratories, Falls Church, Virginia
Dr. S. Salvin, University of Pennsylvania, Pittsburgh

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 8 2/12 | | | 8 2/12 |
| Professional: | 1 5/12 | | | 1 5/12 |
| Other: | 6 9/12 | | | 6 9/12 |

Project Description:

Objectives:

To develop an effective nonliving antituberculosis vaccine from purified fractions of mycobacteria, to study the nature of the host responses to these fractions, and to determine the relationship of delayed hypersensitivity to immunity.

Methods employed:

A variety of physical, chemical and immunologic techniques was used to prepare and characterize cell walls (CW), cell wall skeleton (CWS), and other fractions of several mycobacterial species and strains and to assay the response of experimental animals to these mycobacterial fractions. Protective potency of these preparations was determined in mice, monkeys, and guinea pigs challenged by aerosol with Mycobacterium tuberculosis H37Rv. Histologic examination of various organs was carried out.

Major findings:

1. Vaccine trials in monkeys. Results of previous research at RML suggest that BCG given intravenously (IV) or by aerosol may be more effective than that given intracutaneously (IC). Although IV BCG vaccination would be impractical for human use, aerosol administration of BCG might be feasible. For this reason, members of the RML group (W. R. Barclay and E. Ribi) designed a study in collaboration with the Tuberculosis Panel of the U.S.-Japan Cooperative Medical Science Program, which was conducted under NIH Contract 70-2242 by Dr. Robert Good and his staff at Hazleton Laboratories.

BCG administered IV or by aerosol to rhesus monkeys induced much greater protection against aerosol challenge with virulent human tubercle bacilli than did IC BCG vaccination. The protection afforded vaccinated animals sacrificed and necropsied 3 months after an airborne challenge with 10 to 20 human tubercle bacilli is summarized in the following table.

Summary results of tuberculous disease in BCG-vaccinated rhesus monkeys infected with 10 - 20 VU of H37Rv

| Group | Vaccination route | Vaccine dose (BCG) | Results 3 mo. after challenge (5 mo. after vaccination)* | | | |
|---|---|---|---|---|---|---|
| | | | PPD reaction | X-rays | Pathol. | Culture (H37Rv) |
| A | Intracutaneous | $3.3 \times 10^6$ VU (0.3 mg) | 6/10 | 6/10 | 8/10 | 6/10 |
| B | Intravenous | $3.6 \times 10^6$ VU | 1/10 | 0/10 | 0/10 | 0/10 |
| C | Inhalation | $8.0 \times 10^5$ VU | 3/10** | 1/10 | 2/10+ | 1/10 |
| D | Controls | - | 8/10 | 8/10 | 8/10 | 8/10 |

*No. animals positive/total no. animals

**Reactions were ±.

+ One lesion caused by BCG

Although the aerosol dose of BCG was high, it was only one-fourth that given IC or IV. The aerosol-vaccinated animals showed no adverse side effects; in fact, their chest X-rays remained clear and their tuberculin reactions were only weakly positive. In none of the vaccinated animals did the degree of PPD skin hypersensitivity correlate with immunity.

2. Mechanisms of immunity in tuberculosis. In studies on the mechanism of immunity involved in the vaccination of mice against infection with M. tuberculosis we noted that a vigorous granulomatous response (GR) in the lung was necessary for protection. To determine whether protection could be obtained without GR, we tested a variety of mycobacterial components. In all cases, protection seems to parallel the amount of GR in the lungs. In studying this problem from another angle, we have induced "tolerance" in mice by multiple injections of BCG cell walls in Tween-saline and then vaccinated the "tolerant" animals with BCG cell walls associated with oil droplets. These tolerant animals had a negative footpad test to PPD and had less GR in their lungs than did nontolerant vaccinated animals. However, protection in tolerant animals was even less than that expected from the amount of GR remaining in their lungs. In fact, the protection in some mice with significant amount of granulomatous inflammation in their lungs was no greater than that in unvaccinated control animals. This finding suggests that specific immunity is required for optimal protection, and granulomatous inflammation by itself is not sufficient.

Cell mediated immunity has been associated with the release from lymphocytes of soluble mediators known as lymphokines. Infections with various microorganisms have also been known to result in the release of interferon which may have some relationship to such diverse properties as protection from viral infection and tumor immunity. In collaboration with Dr. S. Salvin of the University of Pittsburgh, we are evaluating various mycobacterial cell walls and components for their ability to release a specific lymphokine, MIF, and interferon. In preliminary experiments, BCG cell-wall immunized animals challenged with PPD released at least as much of these substances as did animals immunized with viable BCG and similarly challenged. The function of these substances in delayed hypersensitivity and tumor immunity will be studied.

3. Components of mycobacteria involved in protection and granuloma formation in mice. We have found that two well-defined components of the mycobacterial CW (see NIAID Project 179) are required to form intense granulomatous inflammation in mouse lungs. Cell wall skeleton-I (CWS-I) is a peptidoglycolipid remaining after vigorous organic solvent extraction of BCG cell walls. This substance alone will not form significant granulomatous inflammation when injected IV into mice. P3, a trehalose ester, is a free lipid obtained from Wax D or cord factor. By itself, it stimulates very little granulomatous inflammation when injected IV into

mice. Neither CWS-I nor P3 will protect mice against an H37Rv aerosol challenge. However, when the two are recombined and injected IV into mice, vigorous granulomatous inflammation results and mice are protected. It appears that P3 amplifies the very slight tuberculin sensitivity of CWS-I and also stimulates granuloma formation, possibly because of its very toxic nature. We have also studied the activity and composition of Wax D and found that the activity in protection and granuloma formation can be explained by the presence of both P3 and a CWS-I-like material. If Wax D is separated into 2 fractions, one of which contains P3, and the other contains the CWS-I-like material, neither by itself has activity. However, when a recombination is made the activity returns. Likewise, P3 is the active lipid in cord factor. CWS-I-like material was also found in some cord factor preparations, and we postulate that cord factor preparations reported in the literature to have activity comparable to Wax D were contaminated with this substance.

4. Adjuvant activity of BCG components. Experimental allergic encephalomyelitis (EAE) appears in animals inoculated with brain antigen emulsified in complete Freund's adjuvant (CFA). We found that BCG cell walls can be substituted very well for the whole tubercle bacilli in CFA. BCG cell walls are often active in doses smaller than 1 µg. Likewise, numerous CW fractions have been found to be active and the common denominator seems to be the particulate CW skeleton. In contrast to its inability to cause granuloma formation, the CWS-I induced EAE. CWS-IV, which is an even more exhaustively extracted skeleton, works as well as the untreated BCG cell wall, whereas it is totally inactive in tumor suppression or in the formation of granulomata in lungs of mice. The common denominator presumably is a particulate material which can carry the basic myelin protein on its surface to the appropriate region of the immune system where cell-mediated immunity is produced. It is apparent that the so-called adjuvant activity of the tubercle bacillus is in reality a function of a number of different molecules which act differently in different adjuvant systems.

Significance to biomedical research and the program of the Institute:

Administration of BCG IC is the generally accepted method for vaccinating man, but its efficacy remains controversial. Previous research in this laboratory with subhuman primates has shown that peripheral routes for both viable BCG and nonviable BCG cell-wall vaccine were inferior to the IV route. The vaccine trial in monkeys closely simulates natural conditions because monkeys were exposed to very small numbers of bacilli which produced a limited, but not overwhelming, disease. Under these conditions of challenge, aerosol vaccination gave protection comparable to that achieved by IV vaccination and both aerosol and IV vaccination provided protection superior to that given by the IC route.

If man responds like the monkey, then aerosol BCG vaccination of man would be superior to the IC route now routinely employed. A further advantage of aerosol administration is the complete absence of side effects often associated with IC administration. All monkeys vaccinated by this route developed local draining abscesses, which also occur in humans vaccinated IC. Furthermore, enlarged regional lymph nodes of man sometimes suppurate and drain.

The relationships among protection, delayed hypersensitivity, cell mediated immunity, and granuloma formation in tuberculosis are still not defined. Likewise, the activities attributed to Wax D and cord factor have been a source of confusion in the literature for years. The adjuvant activity of the tubercle bacillus is of potential importance in the immunization of man against tuberculosis as well as in cancer immunotherapy. All these interacting functions of the tubercle bacillus can only be understood by isolating and identifying the molecular constituents responsible for each, and analyzing them in a variety of test systems. When this has been accomplished, the specific constituents hopefully can be utilized therapeutically, free from the many extraneous side effects of the intact tubercle bacillus.

Proposed course of project:

We will continue to study the mycobacterial fractions which are becoming increasingly more chemically defined. Several fractions now being studied have adjuvant activity and do not induce delayed hypersensitivity to PPD. If we can associate them with an antigen responsible for stimulating immunity against tuberculosis without inducing delayed hypersensitivity to PPD, this achievement will have important implications in the control of tuberculosis and in other theoretical aspects of immunoprophylaxis. Pathologic changes in lungs that might be produced by aerosol vaccination will be investigated. Furthermore, it might not be necessary in humans to give an aerosol dose of sufficient size to produce a skin reaction to 5 TU of PPD. Therefore, dose-response studies are being conducted in subhuman primates vaccinated with aerosolized BCG. Very small numbers of aerosol BCG organisms may still be superior to the IC route of vaccination. Further studies on the granuloma-forming constituents of Wax D and BCG cell walls are under way.

Honors and Awards:

Dr. T. J. Meyer

Invited to participate in a session at the Seventh Annual Tuberculosis Conference sponsored by the Tuberculosis Panel of the U.S.-Japan Cooperative Medical Science Program in Tokyo, August 1972.

Invited to participate in the Conference on the Use of BCG in Therapy of Cancer sponsored by the NCI, Bethesda, Maryland, October 1972.

Publications:

Anacker, R. L., Brehmer, W., Barclay, W. R., Leif, W. R., Ribi, E., Simmons, J. H., and Smith, A. W.: Superiority of intravenously administered BCG and BCG cell walls in protecting rhesus monkeys (Macaca mulatta) against airborne tuberculosis. Z. Immunitaetsforsch. Allerg. Klin. Immunol. 143: 363-376, 1972.

Barclay, W. R., Busey, W. M., Dalgard, D. W., Good, R. C., Janicki, B. W., Kasik, J. E., Ribi, E., Ulrich, C. E., and Wolinsky, E.: Protection of monkeys against airborne tuberculosis by aerosol vaccination with Bacillus Calmette-Guerin. Am. Rev. Resp. Dis. 107: 351-358, 1973.

Anacker, R. L., Matsumoto, J., Ribi, E., Smith, R. F., and Yamamoto, K.: Enhancement of resistance of mice to tuberculosis by purified components of mycobacterial lipid fractions. J. Infect. Dis. 127: 357-364, 1973.

Serial No. NIAID-178
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structure and biologic activity of endotoxins

Previous Serial Number: Same

Principal Investigator: K. C. Milner

Other Investigators: None

Cooperating Units: Dr. William R. Cross, U.S. Army Materials and Mechanics Research Center, Watertown, Mass.
Dr. Jacob Fine, Harvard Surgical Unit, Boston City Hospital, Boston, Mass.
Dr. John Quarles, Michigan State Univ., East Lansing
Dr. J. Robie Vestal, University of Cincinnati, Ohio
Dr. Peter A. Ward, University of Connecticut Medical School, Farmington, Conn.
Dr. Stanley Watson, Woods Hole Oceanographic Institution, Woods Hole, Mass.
Dr. Claes Weibull, University of Lund, Sweden

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 3 9/12 | | | 3 9/12 |
| Professional: | 11/12 | | | 11/12 |
| Other: | 2 10/12 | | | 2 10/12 |

Project Description:

Objectives:

To investigate the features of composition and structure of bacterial endotoxins (ET) required for expression of characteristic host reactivity.

Methods employed:

A combination of biophysical, chemical, and immunological approaches has been used.

Major findings:

1. A new activity of this project involves an evaluation of the Limulus test for endotoxin and efforts to solve some of the problems connected with making the test available to clinical laboratories. Four of the "Cooperating Units" listed above have participated actively in these studies. Although the data obtained to date are to some extent exploratory and preliminary, the following points seem worthy of mention.

a. The extraordinary sensitivity previously reported for the test is, in general, confirmed. Potential hazards in the use of the test, however, are indicated by one earlier report of femtogram sensitivity, which would be incompatible with current knowledge of the chemistry of ET. We have worked with one lysate that regularly detected as little as 8 picograms of ET, and most preparations were clotted by 0.1 - 0.2 nanograms of ET. The latter figures are of the same order as the minimum pyrogenic dose (MPD) for rabbits and about one-tenth of the $LD_{50}$ for 11-day chick embryos inoculated intravenously. Such an amount may be detected in vitro by one tube in a controlled Limulus test. At least 5 rabbits would have to be inoculated with a similar amount before a minimal average rise of 0.6°C could be considered significant. Correspondingly more material would be required for the chick embryo test and still more for other sensitive tests that have been established.

b. The specificity of the Limulus test appears to be equally extraordinary, as illustrated by our work with the synthetic double-stranded polynucleotide, polyriboinosinic-polyribocytidylic acid (poly I-C). Poly I-C is not chemically related to endotoxin in any respect, yet it gives rise to many of the typical reactions to ET in experimental animals. Fever curves produced in rabbits by poly I-C and by ET are superficially similar, but they could be distinguished by a pronounced difference in the lag period between inoculation and onset of fever. The single-stranded polynucleotides from which we prepared poly I-C did not produce fever in doses up to several times the amount of poly I-C that led to pronounced fever, but in very large doses (1 mg or more) they induced fevers of the endotoxin type, indicating minute contamination with ET. Poly I-C would clot Limulus lysate, but only in doses substantially larger than those required to produce fever in rabbits or to kill chick embryos. It is very possible, therefore, that the lysate reacted chiefly to the contaminating ET, rather than to the poly I-C per se. Other evidence for greater specificity has come from observations on anomalous endotoxins from enteric bacteria and from brucellae.

c. Each of six preparations of lysate that were lyophilized and later reconstituted with water retained satisfactory activity. Availability of dried material is considered essential for widespread use of the test.

2. Several investigations from previous years have been continued and another new one has been begun. The latter is in collaboration with Dr. Quarles who is using a simulated kidney machine in which filters of various known pore sizes an be incorporated. We are interested because materials with ET-like properties appear to be traversing filters that were expected to exclude ET. We have also been able to supply Professor C. Weibull with a moderately good set of endotoxic extracts from mutant cultures with progressively deficient content of polysaccharide. He has recently returned electron micrographs demonstrating correlations between morphology and polysaccharide content. In addition, we have honored numerous requests for ET from other investigators, who, in some cases, may engage in future collaborative studies. These continuing investigations have been progressing slowly, and further discussion of them will be reserved for future reports. When it became apparent that Dr. Azuma could be here only one year, it was decided that he should concentrate on study of mycobacterial lipids only. Therefore he has not participated in ET research as projected last year.

Significance to biomedical research and the program of the Institute:

Renewed interest in endotoxins is widespread because of growing acceptance of the views of Dr. Jacob Fine. In brief, these assert that small amounts of ET from the normal gram-negative flora of the gut continuously pass from the lumen into the circulation. In normal individuals, this ET is immediately detoxified by defense mechanisms, both humoral and cellular. In many types of injury and disease, however, increased amounts of ET are mobilized into the circulation when defense mechanisms are concomitantly impaired. Any loss of liver function is especially important in this regard.

The particular advantages of the Limulus test are not only that it is a simple in vitro method, both sensitive and specific, but that it is capable of detecting ET in body fluids, especially serum or plasma. Controversy, of course, continues, but Dr. Fine, Dr. J. Levin, and others have presented evidence that levels of circulating ET in patients have great prognostic significance. Consequently, there is now a great demand for the test in clinical laboratories. What can be done readily in a research laboratory, however, is not easily made foolproof for commercial packaging and distribution. Our work has been undertaken at the request of those who have both an interest in the test and access to horseshoe crabs (Limulus polyphemus) but who need help with standardization of the procedure.

Proposed course of project:

We feel committed to further work on the Limulus test in the coming year. The kind of investigation described above will be enlarged and fortified. One outstanding need is for a reliable ET standard which could be widely distributed as a positive control and as a reference standard for making semiquantitative estimates of ET levels in body fluids. A provisional standard has been packaged here and sent to Drs. Fine and Watson, but this leaves something to be desired. We have arranged to collaborate with Dr. Rudbach in the production and testing of a standard preparation of adequate size. We also intend to continue work here on some of the considerable difficulties that have arisen in suitably packaging a standard for distribution to clinical laboratories.

Other collaborative studies, briefly noted above, are expected to continue. Dr. J. Robie Vestal has received the support of his university for spending six to eight weeks here this summer. We will be working together on the further characterization of lipopolysaccharide antigens from Thiobacillus ferrooxidans cultivated on three different energy sources.

Honors and Awards:

Dr. K. C. Milner

Session chairman and discussion leader, Gordon Conference on Microbial Toxins, Andover, N.H., July 31-August 4, 1972.

Moderator and speaker, Round Table on "The Limulus lysate test for the detection of endotoxin", Miami Beach, May 10, 1973.

Editorial Board member, Infection and Immunity.

Faculty Affiliate (Lecturer in Medical Microbiology), University of Montana.

Publications:

Cutler, J. E., Friedman, L., and Milner, K. C.: Biological and chemical characterization of toxic substances from Candida albicans. Infect. Immun. 6: 616-627, 1972.

In press:

Milner, K. C.: Patterns of tolerance to endotoxin. J. Infect. Dis. Endotoxin Suppl., 1973.

Serial No. NIAID-179
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The role of morphological elements of microorganisms in cancer immunotherapy and related phenomena

Previous Serial Number: Same

Principal Investigator: E. Ribi

Other Investigators: F. R. Abinanti, I. Azuma (NIH Visiting Scientist), T. J. Meyer, K. C. Milner, R. F. Smith, and R. Parker (NIH Postdoctoral Fellow)

Cooperating Units: Drs. B. Zbar, H. Rapp, and T. Tanaka, NCI, NIH
Dr. J. Gutterman, M. D. Anderson Hospital and Tumor Institute, University of Texas, Houston, Texas
Dr. J. E. Sokal, Roswell Park Memorial Institute, Buffalo, N.Y.
Dr. R. Parker and Mr. C. J. Filz, Ivan Sorvall, Inc. Instrument Research Laboratory, Hamilton, Montana
Dr. B. Janicki, Veterans Hospital, Washington, D.C.
Dr. S. Sobol, Bureau of Narcotics and Dangerous Drugs, Department of Justice, Washington, D.C.
Dr. M. Braude, Center for Studies of Narcotics and Drug Abuse, NIMH, Chevy Chase, Maryland
Dr. W. Gentner, Dept. of Agriculture, Beltsville, Md.
Mr. S. M. Strain, University of California, Davis
Miss J. Fulton, University of Montana, Missoula

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 7 10/12 | | | 7 10/12 |
| Professional: | 2 8/12 | | | 2 8/12 |
| Other: | 5 2/12 | | | 5 2/12 |

Contract Support:

| Contract Number | Project Officer (NIAID) | Contractor | Contractor's Project Director | Annual Funding Level |
|---|---|---|---|---|
| NIH-NIAID-73-2513 | E. Ribi | Ivan Sorvall, Inc. Norwalk, Conn. | R. Parker | $39,676 |

Project Description:

Objectives:

Elucidation of the fine structure and chemical composition of microorganisms and correlation of these attributes with certain biologic properties of cells or cell fractions, and the development of new tools and techniques and their application to current problems are primary goals.

Studies this year have been concerned with 1) the isolation and structural identification of mycobacterial cell wall preparations responsible for the enhancement of specific tumor immunity (suppression of growth and regression of established tumors), and 2) applying microparticulate gel chromatography for separating and isolating high molecular weight fatty acids (mycolic acids as methyl esters), prostaglandins, and opium alkaloids.

Methods employed:

A combination of biophysical, biochemical, and immunological techniques has been used, including centrifugal microparticulate gel chromatography (CMGC) and specialized adaptation of the cell fractionator for the preparation of mycobacterial cell walls and disrupted tumor cells.

Tumor immunity induced by mycobacteria and their cellular fractions was studied with a model consisting of a transplantable hepatoma adapted to the ascites form (tumor line 10) in strain 2 guinea pigs. Intradermal inoculation of $10^6$ tumor cells resulted in progressive tumor growth with extensive metastases which caused death of the animals in 60-90 days. Mycobacterial fractions were assayed for their ability to 1) inhibit the growth of tumors accompanied by induction of systemic tumor immunity when injected together with $10^6$ line 10 tumor cells (suppression test), and 2) cause regression of established 7- to 10-day-old intradermal tumors by intratumor injection (regression test).

Major findings:

Both toxic and nontoxic extracts of BCG have been reported to be active in tumor regression and suppression. We have attempted to clarify the issue by systematically studying certain well-defined structural elements of the mycobacterial cell wall both individually and in recombination.

1. The "free lipids" in BCG were most effectively removed by repeated extraction with a series of organic solvents after first deproteinizing the cell walls with a series of proteolytic enzymes. "Free lipids" are defined as lipids removable by treatment with organic solvents alone, without treatment with alkali or mineral acids (see last year's report). The cell wall structure left after removal of "free lipids", termed the cell wall skeleton (CWS-I), is an insoluble polymer complex consisting of a long chain fatty acid, (the mycolic acid(s)), a polysaccharide containing arabinose and galactose units, and a mucopeptide consisting of N-acetylglucosamine, N-glycolyl muramic acid, alanine, glutamic acid and diaminopimelic acid. It also contains a trace of protein whose amino acids are different from those associated with the mucopeptide moiety.

In contrast to untreated BCG cell wall, CWS-I did not induce protection in mice against airborne infection with virulent tubercle bacilli (see report NIAID-177) or regress established tumors, but was highly effective at a low dose level (37.5 µg)in producing systemic tumor immunity in guinea pigs. The presence of "free lipids" in the cell wall was required for tumor regression. As revealed by analytical centrifugal microparticulate gel chromatography (CMGC) the "free lipid" extract contains numerous components in varying quantity, most of which remain unidentified. Attention was directed, therefore, to the widely studied Wax D and cord factor preparations which are "free lipid" subfractions extracted from the tubercle bacillus. These preparations, as prepared according to the original procedures, were mixtures of components, of which some were common to all. With the aid of analytical and preparative CMGC we began a systematic analysis to determine the biological activity of isolated single components, proceeding with those of lowest polarity to the highest. The first active component, designated $P_3$, was identified as trehalose esterified to at least three structurally different mycolic acids. Presumed to be the active principle in classical cord factor preparations, it was highly toxic for mice but, by itself, neither regressed nor suppressed tumors. However, when recombined with CWS-I, the ability of the original cell wall to regress established tumors was restored, as was the ability to protect mice against airborne infection with Mycobacterium tuberculosis H37Rv (see report NIAID-177).

$P_3$ was not the only toxin present in the extract of "free lipids". Another mycolic acid ester of somewhat higher polarity was isolated which possessed some biological activities similar to those of $P_3$. This component, previously unknown, was designated $P_4$ and was found in varying quantities in most of the classical Wax D preparations.

After removal of $P_3$ and $P_4$ from the "free lipid" extract, the material which remained at the top of the chromatographic columns was found to consist predominantly of a mycolic acid-arabinogalactomannan-mucopeptide complex which had structural features similar to those of the CWS-I but was of lower molecular weight. It appeared to be the "nontoxic" adjuvant-active principle of the classical Wax D preparations. In contrast

to the "insoluble" peptidoglycolipid (CWS-I), this "soluble" peptidoglycolipid not only suppressed tumors but also regressed tumors, even though it lacked a detectable quantity of $P_3$. Thus, this fraction can be used in place of the whole cell wall; however, because of its high polarity, it needs further purification which is difficult to achieve with chromatographic procedures. These findings demonstrate the perplexing variety of activities of components of the tubercle bacillus so far studied.

2. Cell walls from M. kansasii were at least as effective as BCG cell walls in regressing established skin tumors in strain 2 guinea pigs. M. kansasii, like BCG, is rich in $P_3$ and also contains "soluble" peptidoglycolipid. Neither finding is true for the saprophytic mycobacteria, such as M. smegmatis whose cell walls were essentially inactive in both tumor regression and suppression tests. In this connection, it is of interest that cell walls of M. kansasii, but not those of M. smegmatis, were as effective as BCG cell walls in protecting mice against airborne infection with virulent tubercle bacilli.

3. Spleen cells from guinea pigs which have suppressed tumor growth and rejected tumors upon challenge (as a result of initial intradermal inoculation of a mixture of 37 µg of BCG cell walls and $10^6$ line 10 tumor cells) were transferred into normal guinea pigs which then rejected a subsequent tumor challenge of the same type. Likewise, immune spleen cells killed line 10 tumor cells ($Cr^{51}$ release) in vitro.

4. Dr. Tanaka, NCI, found that our cell-wall vaccine inhibited the growth of methylcholanthrene-induced T-1038 tumors in C3H/He mice better than did viable BCG. All 20 controls died 70 days after IV inoculation with 5 X $10^5$ tumor cells, whereas 15 of 20 animals that had received the same challenge dose of tumor cells and 150 µg of BCG cell walls were still alive. In a third group that received the same tumor cell challenge and 6 X $10^6$ VU of BCG only 5 of 20 survived. In Dr. Azuma's laboratory at Osaka University, they found that 20 µg of the cell-wall vaccine inhibited growth of melanoma B16 tumor when mixed with 2 X $10^5$ tumor cells and inoculated into syngeneic $C_{57}$ mice. The challenge dose alone leads to progressive growth of a skin tumor, and the animal eventually dies.

5. Other government agencies have been interested in the application of centrifugal chromatography for the identification of opium alkaloids. The major objective is to obtain chromatographic patterns of contaminants or fillers in heroin and morphine street samples, and thereby determine the location of "laboratories" which illegally produced the drugs. We established solvent systems which enabled us, within a few minutes, to separate morphine, codeine, narceine, narcotine, thebaine, papaverine, and noscapine from an artificial mixture.

6. Upon the suggestion of Dr. W. R. Barclay, AMA, we successfully used centrifugal chromatography to separate certain prostaglandins which were difficult to resolve with other methods. In one centrifugation it was possible to separate a mixture of prostaglandins $E_1$, $E_2$, $F_1$, $F_2$, and A. The limit of detection was about 1.0 µg. Isolation of single compounds from this mixture by the preparative pressure elution technique was also done.

Significance to biomedical research and the program of the Institute:

The use of BCG in treating human cancer is being increasingly utilized. The basic pathophysiology of its action is poorly understood and the numerous host-reactive properties of BCG make it difficult, if not impossible, to fully elucidate its mode of action until well-defined molecules are studied. Since the BCG cell wall seems to contain all the properties of viable BCG, it is an excellent source for fractionating and isolating the various molecules active in immunotherapy. There are definite indications in various animal-tumor systems that BCG can enhance as well as retard tumor growth. It is essential, therefore, to determine the actual factors responsible for these actions in order to most effectively utilize BCG in treating human cancer.

Mycobacterial lipids containing mycolic acids play an important role in stimulating cellular immunity, in eliciting granulomatous response and in causing tissue damage. The combination of analytical centrifugal and preparative pressure elution chromatography has made it possible, for the first time, to separate mixtures of mycolic acids with carbon numbers as high as 90. This technique has many applications outside the field of tuberculosis and fills an existing need for an inexpensive, rapid, high-resolution tool for use in forensic medicine, toxicology and pharmacologic studies of drugs.

Proposed course of project:

Continued efforts will be made to isolate the adjuvant properties of the BCG cell wall which make the induction of strong tumor immunity possible. We would like to determine whether delayed hypersensitivity to the tubercle bacillus itself is required for tumor rejection. Ideally, of course, we would like to substitute the tumor specific antigen (TSA) for the tuberculin sensitivity and thus channel the entire immune response toward the TSA. The noxious side effects of tuberculin hypersensitivity would be eliminated and hopefully the cell-mediated immunity toward the TSA would be enhanced by eliminating antigenic competition. We have some evidence that certain mycobacterial fractions that do not sensitize the animals to PPD are effective, at least in suppression of tumor growth. The regression of established tumors by these fractions alone, in combination with $P_3$, will require further study. The *in vitro* cytotoxicity and immune cell transfer systems will be used to quantitate cell-mediated immunity induced

to the tumor cells. With these methods we hope to identify which components produce the most effective "killer" cells and to define the nature of these cells. By utilizing the combination of in vitro and in vivo systems to analyze the action of the various well-defined BCG components, we hope to contribute to the basic understanding of cancer immunity as well as to the practical application of BCG immunotherapy.

To expand our ongoing work with the line 10 tumor cell-guinea pig model, we have initiated experiments to determine whether a correlation between tumor growth and alterations in enzyme activity will be of value in diagnosis and prognosis of the disease. Likewise, the course of tumor regression as a result of treatment with mycobacterial fractions might be followed by assaying iso-enzyme or enzyme changes. Also, the physical fractionation of the line 10 tumor cell into its morphological elements and the elucidation of the antigenic structure of the isolated cell membrane are in progress.

Further work to develop centrifugal and pressure elution chromatography by experimentation with new bed materials and higher pressures is planned. The use of these chromatographic procedures and other techniques for the study of opium alkaloids, prostaglandins, and high molecular weight fatty acid esters will be continued.

Honors and Awards:

Dr. E. Ribi

Continued as member of the Tuberculosis Panel of the U.S.-Japan Cooperative Medical Science Program.

By invitation, presented paper at the Annual Meeting of the Montana Tuberculosis and Respiratory Disease Association, Helena, April 1972.

Served as Chairman of a Session at the Seventh Annual Tuberculosis Conference sponsored by the Tuberculosis Panel of the U.S.-Japan Cooperative Medical Science Program in Tokyo, August 1972.

Invited to participate at the Symposium "New Advances in Analytical Techniques" sponsored by the American Oil Chemists' Society, Ottawa, September 1972.

Invited to participate at the Conference on the Use of BCG in Therapy of Cancer sponsored by the NCI, Bethesda, Maryland, October 1972.

Recipient of the IR-100 Award for 1972,for development of centrifugal microparticulate bed chromatography, sponsored by Industrial Research, Inc.

Dr. I. Azuma

Invited to participate at the Seventh Annual Tuberculosis Conference sponsored by the Tuberculosis Panel of the U.S.-Japan Cooperative Medical Science Program in Tokyo, August 1972.

Invited to participate in the Conference on the Use of BCG in Therapy of Cancer sponsored by the NCI, Bethesda, Maryland, October 1972.

Publications:

Zbar, B., Rapp, H. J., and Ribi, E. E.: Tumor suppression by cell walls of Mycobacterium bovis attached to oil droplets. J. Natl. Cancer Inst. 48: 831-835, 1972.

Ribi, E., Harris, S. C., Matsumoto, J., Parker, R., Smith, R. F., and Strain, S. M.: Pressure accelerated chromatography through microparticulate silica columns packed by centrifugation. J. Chromatogr. Sci. 10: 708-711, 1972.

In press:

Ribi, E., Meyer, T. J., Azuma, I., and Zbar, B.: Mycobacterial cell wall components in tumor suppression and regression. National Cancer Institute Monograph

Ribi, E., Smith, R. F., Strain, S. M., Petcoff, D. G., Parker, R., and Goode, G.: Purification of $\Delta^9$-tetrahydrocannabinol, an active constituent of marihuana, by accelerated microparticulate gel chromatography. Prep. Biochem.

Zbar, B., Rapp, H. J., and Ribi, E.: An experimental model for immunotherapy of cancer. National Cancer Institute Monograph

Serial No. NIAID-180
1. Rocky Mountain Laboratory
2. Epidemiology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The encephalitides

Previous Serial Number: Same

Principal Investigator: L. A. Thomas

Other Investigators: J. E. Coe

Cooperating Units: Dr. David B. Lackman, Microbiology Laboratory, Montana State Department of Health, Helena
Dr. Hugh L. Keegan, University of Mississippi School of Medicine, Jackson

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 2 1/12 | | | 2 1/12 |
| Professional: | 1 | | | 1 |
| Other: | 1 1/12 | | | 1 1/12 |

Project Description:

Objectives:

Major objectives of this project are 1) continuation of study to elucidate the role garter snakes may play in the epidemiology of western equine encephalomyelitis (WEE) virus, 2) to determine if bloodsucking leeches are capable of transmitting WEE or Japanese B encephalitis (JBE) viruses, 3) to determine arbovirus activity in the human and animal population as measured by presence of specific antibody, and 4) to establish identification of field isolates by means of antigenic comparison.

Methods employed:

Standard procedures are used in all phases of study, including preparation of antigens and immune sera, isolation and identification of viruses, and serological tests (hemagglutination (HA), hemagglutination-inhibition (HAI), complement fixation (CF), and neutralization).

Major findings:

1. Study to determine possible role of the water buffalo leech (Hirudinaria manillensis) in the ecology of JBE and WEE viruses. After preliminary viremia studies in rabbits, chickens, and swine employing two strains of JBE virus (Korea-29 and Nakayama) swine were selected for transmission experiments with the Korea-29 strain. Fifteen leeches fed to completion on a JBE-infected swine on the third day after inoculation, when the blood virus titer was $10^{-3.9}$ suckling mouse $LD_{50}$. As determined by viremia and antibody studies, the leeches did not transmit the virus when fed on a normal swine 49 days later. Because the level of viremia in swine inoculated with WEE virus was low, rabbits were used as donor animals. Four leeches, previously fed on the JBE-infected swine, were fed on WEE-infected rabbits when the blood titer range was from $10^{-1.5}$ to $10^{-4.1}$ suckling mouse $LD_{50}$ (fed Feb. 1973). These leeches will be refed on normal rabbits or swine in attempts to demonstrate transmission of WEE and/or JBE virus.

2. WEE and St. Louis encephalitis (SLE) virus activity in Montana. We continued to receive serum specimens from physicians and the Montana State Department of Health for diagnostic serology. In a survey for HAI antibodies against WEE and SLE viruses involving 1,805 sera collected from four counties (Carbon, Phillips, Roosevelt, and Valley) the seropositive rate for WEE varied from 1 to 13% by county, whereas that for SLE varied from 1 to 9%. These viruses did not constitute a public health problem the past year. A sampling of 33 Culex tarsalis and 251 Aedes sp. were free of virus and only 3 sets of paired sera were submitted for tests.

3. WEE virus activity in Washington. Of 46 paired and 3 single human sera submitted by the Washington State Department of Health WEE, CF, and HAI antibodies were detected in 4 of the individuals (paired sera) and 1 of the 3 single sera was HAI positive. Paired sera from 19 horses and 1 single serum were tested: 5 horses with paired sera were HAI positive and CF negative, 9 were both CF and HAI positive, and 2 of the single sera were HAI positive.

4. Antibody response in garter snakes. (See Project NIAID-175).

Significance to biomedical research and the program of the Institute:

It is hoped current studies on antibody formation in garter snakes will provide leads in designing specific tests to detect antibodies in garter snakes following experimental infection with WEE virus.

Proposed course of project:

The study of WEE virus in leeches will be continued. Snakes will be studied for their possible role in the ecology of WEE virus using techniques developed the past year. The laboratory will continue to be of service to the Montana State Department of Health and other agencies in the isolation of virus and serological testing for antibodies.

Serial No. NIAID-181
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Tick-borne disease agents

Previous Serial Number: Same

Principal Investigators: C. M. Clifford, C. E. Yunker, and L. A. Thomas

Other Investigators: J. E. Keirans and R. N. Philip

Cooperating Units: Drs. R. Rausch and Ritter, Arctic Health Research Center, College, Alaska
Dr. Gerald Walton, University of Cork, Ireland
Dr. E. Easton, University of Dar Es Salaam, Tanzania
Dr. J. Casals, Yale Arbovirus Research Unit, New Haven
Dr. J. E. George, Texas Technological College, Lubbock
Mr. R. Gresbrink, Oregon State Board of Health, Portland
Mrs. Margaret Sweeney, Idaho State Dept. of Health, Boise

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 5 | | | 5 |
| Professional: | 1 5/12 | | | 1 5/12 |
| Other: | 3 7/12 | | | 3 7/12 |

Project Description:

Objectives:

To determine the incidence, distribution and ecology of tick-borne diseases that influence man and domestic animals in the United States. Of equal importance is the investigation of the vector relationships of the increasing number of viruses known or thought to be associated with ticks.

Methods employed:

Field and laboratory approaches include conventional and specialized entomological, ecological, virological, and immunological techniques.

Major findings:

1. Viruses and ixodid ticks. The identification of 16 isolates obtained in 1971 from Ixodes uriae in western North America was continued. Three viruses previously recognized from northern Oregon, Yaquina Head, "86" (Sakhalin-like) and Uukuniemi, were identified from ticks collected on offshore island of southern Oregon. Of two viruses isolated by the Arctic Health Research Center from I. uriae in mainland Alaska and sent to us for identification, one reacted in complement fixation (CF) test with antisera from Sakhalin and "86" viruses (ungrouped) but failed to react in plaque reduction or suckling mouse neutralization (N) test with either of these antisera. Further tests are planned. The other was indistinguishable from Great Island virus (Kemerovo group) by CF and N tests. Great Island virus, presently undescribed, previously has been recovered from I. uriae in Newfoundland; ours is a new geographic record. Eleven additional isolates collected from this species in Oregon in 1971 were mixtures of "86", Yaquina Head or Uukuniemi viruses, but were too low in concentration to identify and may represent an undescribed virus.

I. uriae collected in murre nesting sites along the Oregon Coast in 1972 were tested as 143 pools in baby mice and Vero cells. Of 15 strains of virus isolated 14 were identified in a CF screen test as "86" virus and one as Yaquina Head virus. This year we tested ticks in pools of smaller sizes and as individuals to lessen the possibility of obtaining isolates of mixed identity and, with engorged ticks, to avoid neutralization of these viruses by host-produced antibody. It also allows us to derive some significant statistical inferences concerning infection rates in various stages and sexes of ticks. The observed infection rate of 44 female ticks tested individually was 6.8%. A probability estimate of male infection rate, based on testing ticks in 125 pools of five ticks each, was approximately equal (6.4%). Since the opportunity for nymphs (destined to become males) to ingest and retain virus is equal to that of female nymphs and, since adult males are not blood feeders, the comparable infection rates indicate transstadial transmission of the virus. When females were tested in pooled lots, "86" virus could not be recovered, suggesting interference from other agents. This Sakhalin-like virus was also recovered from three pools of unfed larval ticks; this finding strengthens earlier evidence that the virus is capable of transovarial passage. Results of a serological survey of coastal Oregon residents for infection with tick-borne viruses mentioned above suggest that "86" virus may be infective for man (see NIAID-182).

Thirty-eight sera from young Sooty and Noddy terns of the mid-Pacific area were tested in plaque reduction neutralization test (PRNT) in Vero cells against Yaquina Head and Uukuniemi viruses. Results were negative.

I. howelli, a common parasite of the Cliff Swallow, was collected in the Sapphire Mountains near Philipsburg, Montana, where this tick had previously yielded the first isolates of Sapphire I, an ungrouped arbovirus. Forty-seven pools of these ticks were tested in baby mice and Vero cell cultures with negative results.

Our collaborative study with Oregon and Idaho State Departments of Health was continued. Ixodid ticks from various localities in Oregon were tested for pathogens throughout the tick season and 2 isolations of Colorado tick fever (CTF) virus were made from 194 pools. Thirty-four blood samples from Idaho residents with fever and history of tick bite were tested in baby mice and Vero cells. From these, 7 isolates of CTF virus were made. Four were obtained only in suckling mice after blind passage of mouse brain. However, refrigerated samples of 6 of these positive bloods were retested in Vero cells 4 months later, using ultrasonic vibration to disrupt red cells; all were positive and, in each case, plaque numbers were significantly increased (up to 20-fold) over that originally observed.

When we reported the isolation of Tuleniy virus, we also indicated its close serological relationship to Japanese B virus. Because of this we attempted to determine if our Oregon strain of Tuleniy virus could be transmitted by a vector of Japanese B encephalitis virus (JBE). We have demonstrated that Culex tritaenorhynchus could acquire but not propagate Tuleniy virus. To validate this experiment it was necessary to determine if C. tritaenorhynchus from our colony could transmit JBE virus. Preliminary experiments, which indicate that our reared mosquitoes retained adequate levels of virus necessary for the transmission of this virus, were interrupted due to the death of our insectary attendant.

2. Viruses in argasid ticks. The confused classification of Hughes group agents, which we are isolating in quantity from North American ticks, has prompted us to investigate the antigenic relatedness of North American strains by CF and PRNT tests. The latter are still in progress. CF cross-box titrations showed that Sapphire II and Soldado viruses are completely separable from each other and from Hughes, Raza, Punta Salinas, and Farallon viruses. Farallon virus is related to but readily separable from Hughes, Raza, and Punta Salinas viruses by reciprocal CF test, and separable from Hughes virus by PRNT.

Progress in identifying tick-borne viruses, particularly of the Hughes group, is impeded by their low pathogenicity in conventional animal and cell culture systems, their poor plaquing capability, and by low reactivity of their antisera. By prolonging the incubation period of virus-infected, agar-overlayed Vero cells, all of the above Hughes group agents

have been plaqued. In addition, a method was devised to increase the avidity of the neutralizing reaction. In this procedure virus-antiserum mixture, which is normally incubated at 37°C for 1 hour, is held at 4°C for an additional 24 hours before inoculation. In controlled tests, the reduction of Farallon virus by a variety of antisera to Hughes group agents was tested in Vero cells before and after refrigeration. Nearly 1 1/2 dex increase in neutralization was seen for the homologous system, but not the heterologous ones, when the extra step was employed. In mixtures not so treated, the homologous system was indistinguishable from heterologous systems and from normal mouse serum. Subsequent additional experiments with many other viruses have confirmed the validity and broad applicability of this procedure.

Two hundred and eighty-five Argas cooleyi, parasites of Cliff Swallows collected in New Mexico, were sorted by sex and stage into pools and inoculated into baby mice and Vero cell cultures. From these 36 strains of virus were isolated, of which 13 proved to be Sixgun City virus and 23 Sapphire II virus. The former is a Kemerovo group agent, the latter a member of the Hughes group. One locality, from which a large series of ticks were tested individually, was apparently virus-free while 14 to 30% of nymphal and adult ticks collected in a nearby area yielded one or both viruses.

Three isolations of a virus were made in Vero cells from Ornithodoros coniceps maritimus collected by Dr. G. Walton at a seabird nesting site off the coast of Ireland. These isolates reacted to Soldado antisera in CF and PRNT. This virus, first reported from terns and ticks in Trinidad, BWI, had been recovered subsequently only from O. capensis in inland Ethiopia. Thus, this is a new record for the Palearctic region. The related Zirqa virus from O. musebecki is known to cause human illness in the Arabian Gulf.

3. Assay systems for tick-borne viruses. Ten viruses were added to our bank of tick-borne pathogens this year. These are: Bauline, Dhori, Great Island (strain 41), Great Island (strain 45), Hazara, Jos, Louping Ill, Mut, Royal Farm, and Upolo. Virus stocks, antigens, and antisera were prepared for most of these. We continued to develop CPE and plaque assay systems in Vero, BHK-21, and PK-H13 cells for many of these agents. Of particular importance was the successful plaquing of Hughes group agents, which allows us to identify and characterize the numerous strains of this group that we are continually finding in our field-collected ticks or in ticks sent to us by other investigators.

Significance to biomedical research and the program of the Institute:

The genus Ixodes plays an important role in the transmission of viruses that cause illness in man, e.g., RSSE, Central European, Louping Ill, Kemerovo, Omsk haemorrhagic fever, and Powassan virus. They are also important vectors of pathogenic viruses to domestic livestock. Our demonstration of a multiplicity of viruses in Ixodes ticks in North America is of potential epidemiological significance.

Again this year our findings emphasize the tremendous distribution of viruses associated with argasid and ixodid ticks, i.e., the occurrence of Soldado virus from islands near Ireland and the presence of Great Island virus (known only from New Foundland) from I. uriae in Alaska. Preliminary serological results suggest that "86" virus (related to Sakhalin virus) may be infective for man. If this is true the high infection rate demonstrated this year in ticks would provide an excellent source of virus for human infection, if and when the tick ecosystem is invaded by man.

Progress in identification of tick-borne viruses, particularly of the Hughes group, has been impeded by the low pathogenicity they produce in suckling mice, their poor plaquing capacity and the low reactivity of the antisera produced against them. The improvement of techniques that will allow Hughes group viruses to plaque, as well as improved neutralization test procedures, increase the facility for recognition and characterization of these viruses.

During the year we have added ten viruses to our bank of tick-borne pathogens. The RML now has the capability of identifying tick-borne viruses from most areas of the world.

Proposed course of project:

Isolation and characterization of viruses associated with ticks will continue. Emphasis will be placed on devising techniques to allow for antigenic comparison of viruses now on hand. We will continue to add to our reference stock of tick-borne and related viruses and to prepare serological reagents for these agents. Efforts will be continued to develop tissue culture techniques to facilitate identification of tick virus strains as they are recovered.

Hopefully we will be able to determine the vector potential of Culex mosquitoes for viruses isolated from North American ticks. However, full scale resumption of activity and successful completion of these studies must await the replacement of an attendant in the RML insectary.

Publications:

Yunker, C. E., Clifford, C. M., Thomas, L. J., Cory, J., and George, J. E.: Isolation of viruses from swallow ticks, Argas cooleyi, in the southwestern United States. Acta Virol. 16: 415-421, 1972.

In press:

Thomas, L. A., Clifford, C. M., Yunker, C. E., Keirans, J. E., Patzer, E. R., Monk, G. E., and Easton, E. R.: Tick-borne viruses in western North America. I. Viruses isolated from Ixodes uriae in coastal Oregon in 1970. J. Med. Entomol.

Yunker, C. E., Clifford, C. M., Keirans, J. E., Thomas, L. A., and Cory, J.: Tick-borne viruses in western North America. II. Yaquina Head, a new arbovirus of the Kemerovo group isolated from Ixodes uriae. J. Med. Entomol.

Serial No. NIAID-182
1. Rocky Mountain Laboratory
2. Epidemiology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The epidemiology of human infections of special interest

Previous Serial Number: Same

Principal Investigator: R. N. Philip

Other Investigators: E. A. Casper, L. A. Thomas, and C. E. Yunker

Cooperating Units: Dr. D. Francis, EIS Officer, Oregon State Division of Health, Portland

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 1 3/12 | | | 1 3/12 |
| Professional: | 6/12 | | | 6/12 |
| Other: | 9/12 | | | 9/12 |

Project Description:

Objectives:

Studies in this project acquire basic information about the epidemiology and prophylaxis of human infection by agents of special interest to RML.

Methods employed:

Field investigations utilizing laboratory methods for isolating disease agents and detecting antibody responses to infection are emphasized. The methods are standard epidemiologic, statistical, and diagnostic procedures. As the situation warrants, specialized laboratory techniques are used for diagnosis.

Major findings:

1. Colorado tick fever (CTF)

a. Immunofluorescence (FA) as a diagnostic procedure in CTF infection of man. Using FITC-conjugated antibody (hamster), we evaluated direct FA in the detection of intra-erythrocytic CTF antigen. Clotted blood samples, which were submitted by local physicians in 1971 and 1972 from 50 patients suspected to have CTF, were held at 4°C for 3-18 months and examined for CTF virus by suckling mouse inoculation and direct FA. Nineteen of 34 specimens positive in mice were also positive by FA whereas all of 16 negative in mice were also negative by FA. Thus, the FA test was less sensitive than the mouse system in detection of CTF virus. However, this difference was partly due to the time specimens were obtained, because parasitized erythrocytes were infrequent before the 5th day of illness. Of those positive in mice, only 4 of 17 obtained during the first 4 days of illness were positive by FA whereas 15 of 17 obtained from 5 days to 6 weeks after onset were also FA positive.

An indirect FA test using CTF (Florio) infected Vero cells as antigen was devised to detect CTF antibody. Of 186 sera from candidate CTF patients, 63 (34%) had FA antibodies ranging in titer from 1:8 to 1:4096 and only 24 (13%) had CF antibodies in titers of 1:8 to 1:64. In no instance was the CF test positive in absence of FA antibody. Paired sera were obtained from 12 patients from whom CTF virus was isolated. Ten had $\geq$ 4-fold rises in CTF-FA antibody titers during convalescence. None of 6 virus-negative patients had CTF antibody responses.

b. Stability of CTF in clotted blood. The remarkable stability of CTF virus in erythrocytes held at 4°C was evidenced by the success in re-isolation of virus from clotted blood obtained from CTF cases in 1971. Using sonication to disrupt the stroma of washed cells, virus was re-isolated from 9 of 10 specimens shown to be positive 14 to 16 months earlier.

c. Laboratory-acquired CTF. A laboratory-acquired case of CTF presented an unusual opportunity to clarify virus-host relationships in human infection. Typical clinical CTF with a bimodal fever curve occurred in a laboratory technician 4 days after a contaminated-needle prick. Viremia in the plasma fraction was detected 20 hours before onset of symptoms and persisted until the 9th illness day, reaching maximal titers of 3.0 $\log_{10}$ Vero-cell plaque-forming units on the day of onset. Virus in the cellular fraction was first detected 24 hours after onset of illness. Maximal titers (5.4 $\log_{10}$ plaque-forming units) were attained on day 10 and virus could still be recovered on day 82. Parasitized erythrocytes were first seen by direct FA on day 3. They reached maximal numbers (100 per 5 million rbc) during the 2nd week of illness and antigen was last detected in erythrocytes on day 124. Appearance of FA antibodies coincided with disappearance of virus from the plasma. The peak antibody titer of 1:1024 was reached on day 17 and a titer of 1:512 was still observed at

6 months. CF antibodies, which appeared late (day 26) and achieved levels of only 1:8, were still detectable at 6 months (1:4). Serum neutralizing activity was first noted on day 9 and coincided with disappearance of virus from the plasma. Peak titers were observed 40 days or later after onset of symptoms.

2. Other tick-borne viruses: A preliminary attempt was undertaken to determine whether an indirect FA procedure might be useful in serotyping tick-borne viruses. The theoretical advantage of such a procedure is that the same drop of serum dilution can be simultaneously reacted against as many as 9 different antigens. The test antigens were Vero cells infected with several strains of viruses isolated from ticks collected off the Oregon coast including Sakhalin, Uukuniemi, Yaquina Head, and Farallon group. The patterns of reaction in cross tests of mouse hyperimmune ascitic fluids corresponded to those obtained in CF tests except that titers were generally 2- to 10-fold higher by FA. The 4 groups of viruses were completely distinct, one from another.

A sero-survey of Oregon residents was conducted to determine whether infection by the aforementioned viruses occurs to any significant degree. Sera from 397 residents of 7 coastal counties submitted to the Oregon Division of Health for routine syphilis serology were sent to us by Dr. Francis for testing against Sakhalin, Uukuniemi, Yaquina Head, and Farallon group viruses. Two showed low reactivity (Sakhalin group, 56300-86 strain) by FA which could not be confirmed by CF or neutralization tests. All others were negative.

Significance to biomedical research and the program of the Institute:

The direct FA test has certain advantages over standard isolation procedures in the diagnosis of CTF, which would be particularly important to the diagnostic laboratory with limited facilities. The test is reliable. CTF antigen is readily recognized and not easily confused morphologically or tinctorially with non-CTF elements in smears of RBC's. The test can be performed and reported within hours of receiving specimens, which is particularly important to the practicing physician. It is easily performed and requires only a source for conjugated antibody. The one disadvantage is that the procedure may frequently be negative in tests of specimens taken before the fifth day of illness.

The fact that viable CTF virus circulates for long periods within RBC's raises new questions as to the significance of this disease as a health problem in the Rocky Mountain area. For example, if the incidence is sufficiently high, CTF could be a problem to recipients of whole blood transfusions. At any rate, a more satisfactory serologic survey procedure is needed to determine the prevalence of infection in this area. The indirect FA test appears to satisfy many of the requirements for such procedure. It may also be useful for examining the serologic homogeneity of strains.

The survey of Oregon coastal residents gave no indication that the viruses recovered from ticks obtained from seabird rookeries were definitely involved in human infection. However, exposure of man to these agents if they are confined to the basic tick-seabird-tick cycle of maintenance would be a rare event unlikely to be detected in a small, indiscriminate sero-survey of human sera. Our chief concern was whether such agents might extend into other vectors more intimately associated with man. There is no evidence that this has occurred to a significant degree.

Proposed course of project:

As time and opportunity permit we will attempt to obtain more complete information on the frequency of CTF in this area by sero-survey using the indirect-FA test. Information will be obtained from blood donation centers such as the Red Cross on unexplained febrile illnesses in recipients of transfusions, particularly during summer and fall months. If CTF appears to be a problem, this possibility will be explored further.

Serial No. NIAID-183
1. Rocky Mountain Laboratory
2. Slow Viral Diseases Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Viruses as a probable cause of certain chronic diseases of man

Previous Serial Number: Same

Principal Investigator: W. J. Hadlow

Other Investigators: M. E. Bloom, B. W. Chesebro, J. E. Coe, T. A. Jackson, D. L. Lodmell, J. J. Munoz, and R. E. Race

Cooperating Units: USDA Animal and Plant Inspection Service, Veterinary Services: Dr. A. L. Klingsporn, Hyattsville, Md., and Mission, Texas
Dr. C. M. Eklund, Hamilton, Montana (consultant)

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 16 1/12 | | | 16 1/12 |
| Professional: | 2 10/12 | | | 2 10/12 |
| Other: | 13 3/12 | | | 13 3/12 |

Project Description:

Objectives:

From a study of naturally occurring slow viral diseases of domestic animals, 1) to obtain an understanding of the mechanisms by which viruses cause protracted diseases, and 2) to develop working concepts and investigational procedures that will facilitate determining whether viruses are likewise causally related to some chronic diseases of man.

Methods employed:

Four diseases that are the models for continued study include: 1) scrapie, a subacute polioencephalopathy of sheep, 2) a comparable neurologic disease of ranch mink, 3) Aleutian disease, a protracted systemic disease of ranch mink, and 4) progressive pneumonia, a chronic lymphoreticular proliferative pulmonary disease of sheep. Clinicopathologic, virologic, and immunologic features are studied to gain an understanding of the natural history and pathogenesis of each disease. Study of the causative viruses is of secondary concern.

Major findings:

1. Scrapie

a. The disease process. Additional virologic data obtained during the past year provide a clearer picture of the natural disease in Suffolk sheep. Special attention was directed to events that occur early in the infection. Thus, by mouse inoculation, virus was detected in each of two clinically normal 11-month-old lambs obtained from the naturally infected flock at the Scrapie Field Trial. It was found in the upper colon and in the suprapharyngeal, prefemoral, and mesenteric-portal lymph nodes of one. In the other, virus was present in the suprapharyngeal, prescapular, prefemoral and mesenteric-portal lymph nodes and in the spleen, ileum, and upper colon. One of three 2-year-old clinically normal Suffolks also was infected. In this sheep, virus not only was widespread in lymph nodes and intestinal tract but was present also in the spleen and brain (medulla oblongata and diencephalon). Presumably, the infection in this sheep was within 3 to 5 months of becoming evident clinically. To obtain more information on the early phase of the infection, tissues from several 3- and 8-month-old lambs as well as other yearlings are being assayed for virus.

These findings provided added support for our view that the intestinal tract and associated lymph nodes are the early sites of viral replication in the natural disease. To what extent the infection remains at this non-neural level is not known. Doubtless, once virus reaches the CNS, disease inevitably supervenes. Whether disease represents the exception in a flock containing many sheep infected at the non-neural level can only be surmised at this time. In view of what occurs in most acute viral infections, this pattern seems likely in scrapie. To obtain some information on this aspect of the natural history of scrapie, key tissues (intestine, spleen, and lymph nodes) of 15 clinically normal 4- to 6-year Suffolk sheep from high risk families are being assayed for virus.

Because we have no evidence that the ovine fetus or lamb at birth is infected in flocks in which scrapie is endemic, a study is underway to determine whether the fetus will support growth of the virus, and if so, what effects this might have on the fetus. Possible immunologic responses

of the fetus to scrapie virus are also being considered. Twenty-three Suffolk fetuses 90-100 days old were inoculated intramuscularly with suspensions of brain (of known virus titer) from Suffolk sheep affected with the natural disease. Apart from three fetuses that died shortly after inoculation, all developed normally and were born alive. At birth and at monthly intervals thereafter, two or three lambs are being killed and their tissues assayed for virus by mouse inoculation. Results from this study will complement virologic data obtained from the examination of lambs born in a naturally infected flock. Among other considerations, the information will allow us to determine whether an eclipse period occurs that could explain our failure to detect virus in fetuses and newborn lambs from a naturally infected flock.

b. The virus. As mentioned last year, suspensions of brain from one Cheviot and two Suffolks affected with natural scrapie were inoculated intracerebrally into pastel mink. A neurologic disease resembling encephalopathy (EN) occurred only in mink that received the suspension of brain from one of the Suffolks. Whether this result reflects differences in the virus titer or inherent variations in the pathogenicity for mink of the three inocula is not known. To obtain more information on this, brain suspensions of known virus titer from two other Suffolks and one Montadale were recently inoculated into mink.

Serial passage of scrapie virus in pastel mink is also underway to evaluate the effect of such passage on the pathogenicity of the virus for the mouse. Workers at Purdue University reported that one passage of scrapie virus in mink made it nonpathogenic for the mouse. If this is so and if EN virus is in fact a variant of scrapie virus as present evidence suggests, then the failure of EN virus to cause disease in the mouse might be explained on this basis.

2. Mink encephalopathy

a. The disease process. Virologic data accumulated laboriously over the past several years now clearly indicate that the pattern of the infection in mink is much like that of experimental (and probably natural as well) scrapie virus infection in the goat. Thus, during the long incubation period, replication of virus in peripheral tissues is confined to lymph nodes, and the titers reached are generally low. Large concentrations occur only in the central nervous system, which becomes infected about 20 months after the mink is inoculated subcutaneously. Spread of virus to a few other non-neural tissues, such as spleen and liver, occurs only after the central nervous system becomes infected. No regular exit of virus from the mink is apparent. Although still incomplete, these findings bear out our earlier contention that EN virus infection in mink, like scrapie virus infection in the goat, is a dead-end one. Mink, therefore, probably are not the natural host of EN virus, and their

infection is accidental. These data also are consistent with the failure of the infection to become established in a herd of mink. As yet unanswered is the important question of the original source of EN virus, which epidemiologic observations indicate was present in the mink's feed.

b. The virus. One passage of EN virus in the goat, in which 10 of 19 became affected with a neurologic disease closely resembling experimental scrapie in this host, did not change its pathogenicity for mink or mice. The goat passage virus caused typical EN in mink and was still without effect on the mouse. Unlike the goat, the Cheviot sheep is rather resistant to EN virus; only 1 of 19 became affected with a similar neurologic disease. Perhaps the Suffolk breed, in which natural scrapie usually occurs in the United States, is more susceptible and may select for virus that is pathogenic for the mouse.

3. Aleutian disease (AD)

a. The disease process. In a study now essentially completed, the response of pastel mink to the Pullman isolate became much clearer. As evidenced by the occurrence of viremia with or without a slight rise in serum gamma globulin, 17 of 20 became infected when inoculated subcutaneously. Disease, however, occurred in only 4 of the 17, even though all of them received an amount of virus that would have killed every similarly inoculated sapphire. When examined more than two years after inoculation, only 1 of 7 infected, but not diseased, mink had virus persisting in the mesenteric node and spleen. Viremia and a rise in gamma globulin seem to be a good measure of the mink's susceptibility to AD. If a rise in gamma globulin does not occur, the mink is resistant to the disease. To determine whether those pastels that resisted the Pullman isolate are either sensitized or resistant to a more virulent isolate of AD virus, 7 remaining mink, 6 that had evidence of infection and 1 that did not, were challenged with at least 10 $LD_{50}$ (as determined in pastels) of the more virulent Utah (Porter) isolate. Results are not yet available.

b. Studies on immunoglobulins and immune responses of normal and AD affected mink. Considerable time, effort, and animals were devoted to these studies whose results are reported under NIAID Project 175.

c. The virus. Comparative studies with the Pullman and Utah isolates provided additional data indicating distinct biologic differences between them. The most striking difference, briefly mentioned last year, concerns the virulence of the Utah isolate for pastel mink. In a parallel titration of this isolate (5th passage), the titer was $10^{-4.8}$ in sapphires and $10^{-5.0}$ in pastels. This unexpected finding is in sharp contrast to the results of comparable titrations of the Pullman isolate. Because of the erratic response (in terms of the occurrence of disease) of pastels to this isolate, a meaningful $LD_{50}$ can not be calculated. For this reason, pastels can not be used in titrating the Pullman isolate. Apparently,

this is not so for the Utah isolate. Nevertheless, the sapphire continues to be the standard animal here for the detection and titration of all isolates of AD virus.

Efforts to establish AD virus in a tissue culture system were continued on a small scale. Cells derived from explants of various tissues of AD-affected mink were passed serially without apparent cytopathic effect. To determine whether virus was present in such cultures, sapphire mink were inoculated with preparations (cells and fluid) from the 4th, 7th, and 10th tissue culture passages. To date, virus has been detected in the 4th passage preparation of bone marrow cells of a pastel mink infected with the Utah isolate. Sufficient time has not elapsed to know whether virus is present in the other passages of this preparation or in similar passages of cells derived from explants of other tissues of mink infected with either the Pullman or Utah isolates. Whether the virus detected in the 4th passage bone marrow cells represents replication in vitro or only original virus in the explant remains to be determined. Regardless, this preliminary finding is the first encouraging result obtained in our many attempts to grow AD virus in vitro.

Other studies on AD virus are reported under NIAID Project 175.

4. Progressive pneumonia

a. The disease process. No new findings can be reported now. The pilot study mentioned last year on pathogenesis was started. Eleven goats were inoculated intravenously with an infectious suspension of sheep lung. Periodically, they are being examined for hematologic changes, viremia, and antibodies. Beginning one year after inoculation, they will be killed at 3- to 6-month intervals and their tissues assayed for virus in tissue culture. A special effort is being made to determine the cellular localization of virus and the role of immunologic phenomena in the initiation and evolution of the seemingly uncontrolled proliferation of lymphoreticular cells in the lungs.

Three sheep and eight goat fetuses were inoculated intracerebrally with a suspension of progressive pneumonia virus to determine whether animals of this age are susceptible. All kids and lambs born will be kept under observation for the occurrence of either neurologic or pulmonary disease.

b. The virus. Some preliminary studies are reported under NIAID Project 175.

5. Incidental observations on mink. In the conduct of this project, many mink are kept under individual clinical observation for long periods and then examined in detail at necropsy. This procedure has provided us with a unique opportunity to obtain much useful information on the general pathology of a species that only recently has become a laboratory animal.

As time permits, this information is being evaluated. Some of the more significant findings include:

A slowly progressive fatal lymphoreticular proliferative disease with some histologic stigmata of Hodgkin's disease has been observed only in sapphire mink. This finding parallels the lymphoma-like condition that commonly supervenes in children affected with the Chediak-Higashi anomaly, to which sapphire mink represent the animal counterpart.

Lymphocytic lymphoma has been found in both sapphires and pastels but occurs most often in pastels 4 to 7 years old. It assumes several macroscopic organ patterns and may be accompanied by leukemia.

Nonlymphomatous neoplasms have also been found in both color phases. Of these, hepatoma, pancreatic adenocarcinoma, and ovarian carcinoma have been the most common ones.

Amyloidosis is commonly found, especially in older pastels. Almost always unassociated with some chronic inflammatory process, it has a distinctive pattern in which the gastric mucosa, submandibular salivary glands, pancreas, and liver are mainly affected.

A condition best described as chronic atrophic gastritis occurs in high incidence in both color phases. Its cause and significance are not known.

Significance to biomedical research and the program of the Institute:

As is evident from requests to us for papers on slow viral diseases and from editorials and other comments in the biomedical literature, interest in "viruses as a probable cause of some chronic diseases in man" is gaining momentum. Yet for various reasons, not many investigators willing to risk a head on approach are attracted to the problem. This project, however, continues to provide a suitable starting point. Although limited to studies on animal models, it nevertheless is helping to define the mechanisms that most certainly operate in the evolution of some chronic diseases of man. Apart from contributing such background information, this project not only is providing a factual basis for a broadened view of viruses as mammalian pathogens, but also is uncovering pathogenetic processes more subtle than those previously associated with animal viral infections. Burnet and White (Natural History of Infectious Disease, 4th edition, Cambridge University Press, 1972) have aptly described the situation. " 'Slow virology' — the major intellectual challenge of infectious disease for the final decades of this century."

Proposed course of project:

The project will continue to pursue its objectives despite present and anticipated reductions in personnel and money. Animal work will remain the core of the project. The extent of collaborative studies no doubt will vary with the enthusiasm of the other investigators for an exciting but difficult research problem. (This project is no place for the "quick kill", which often seems to be sought.)

Contemplated studies include the following:

1. Because the relationship of scrapie and EN viruses is still unsolved, comparative studies will continue along several lines.

2. To better understand the temporal virologic features after a natural route of exposure to AD virus, a sequential study in sapphire mink inoculated orally will be initiated.

3. Titration of the Utah isolate of AD virus by the oral route in pastels will be done to provide a more accurate assessment of the virulence of this isolate under simulated natural conditions in a genotype that is seldom affected by explosive outbreaks of AD.

4. Tissue culture studies with AD virus will be pursued on a larger scale. Developing an in vitro method for detecting and titrating the virus is still a concern of the project.

5. By the use of various special techniques, information will be sought on the mechanism of the lymphoreticular cell proliferation in the lungs of goats inoculated with progressive pneumonia virus.

Honors and Awards:

Dr. W. J. Hadlow

Elected to active membership in the American Association of Neuropathologists.

Continued as Adjunct Professor of Veterinary Pathology, Washington State University, Pullman, Washington.

Invited to present paper on "Myopathies in Animals" at the Symposium on Comparative Pathology of the Heart to be held in Boston September 17-19, 1973. (Accepted)

Publications:

Eklund, C. M. and Hadlow, W. J.: Slow viral diseases. In Bond, R. G. and Straub, C. P. (Eds.): Critical Reviews in Environmental Control. Cleveland, Ohio, Chemical Rubber Company, 1972, vol. 2, pp. 535-555.

Hadlow, W. J., Race, R. E., and Jackson, T. A.: Lymphoreticular proliferative disease in mink homozygous for the Aleutian gene. J. Natl. Cancer Inst. 49: 1455-1457, 1972.

Hadlow, W. J.: Animal models of slow viral disease. In Day, S. B. and Good, R. A. (Eds.): Membranes and Viruses in Immunopathology. New York and London, Academic Press, Inc., 1972, pp. 381-396.

Lavelle, G. C.: Large fraction of scrapie virus resistant to lipid solvents. Proc. Soc. Exp. Biol. Med. 141: 460-462, 1972.

In press:

Hadlow, W. J.: Myopathies of animals. In The Striated Muscle, IAP Monograph #12, Baltimore, Williams and Wilkins, 1973.

Eklund, C. M. and Hadlow, W. J.: Slow viral and related diseases. In Hubbert, W. T., McCulloch, W. F., and Schnurrenberger, P. R. (Eds.): Diseases Transmitted from Animals to Man (ed. 6)

Eklund, C. M. and Hadlow, W. J.: Implications of slow viral diseases of domestic animals for human disease. Medicine

Lavelle, G. C.: Multiplicity of scrapie virus in infected mouse spleen cells in vivo. Infect. Immun.

Serial No. NIAID-184
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biological properties of Bordetella pertussis antigens

Previous Serial Number: Same

Principal Investigators: J. J. Munoz and R. K. Bergman

Other Investigators: J. F. Bell, M. E. Bloom, W. J. Hadlow

Cooperating Units: Dr. Alan Blaskett, Commonwealth Serum Laboratories, Parkville, Victoria, Australia

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 4 8/12 | | | 4 8/12 |
| Professional: | 1 7/12 | | | 1 7/12 |
| Other: | 3 1/12 | | | 3 1/12 |

Project Description:

Objectives:

1) To purify and fully characterize biologically active substances from B. pertussis with special reference to the histamine sensitizing factor (HSF), the protective antigen (PA) and the antibody stimulating substance (adjuvant) of these cells. 2) To study the mechanism of action of HSF, PA, and the adjuvant action of B. pertussis.

Methods employed:

Standard immunological, pharmacological, biochemical and bacteriological techniques and physicochemical methods of fractionating biological material are used.

Major findings:

1. Purification of HSF (Munoz). Techniques have been devised to obtain HSF in a reasonable degree of purity. Acetone-treated cells are extracted with 1 M NaCl containing 0.05 M Na pyrophosphate at pH 8.5. The extract (BPE) is dialyzed and passed through a hydroxylapatite column which retains the HSF and many other antigens found in the crude extract. The fractions were eluted stepwise with .01 M, .1 M, .2 M, and .65 M phosphate buffer at pH 6.2. Most of the HSF is in the 0.2 M phosphate buffer eluate, although some is also in the 0.65 M eluate. The 0.2 M fraction also contains 5 or 6 other antigens of which all but 2 can be removed by precipitating the HSF at pH 4.5. The last 2 contaminants were removed by electrophoresis in agarose. The final product is fully active and contains no demonstrable antigens when tested with hyperimmune sera made in rabbits against BPE or the active 0.2 M fraction from the hydroxylapatite column. Enough of this material will be collected to determine the chemical nature of HSF.

2. Relationship of HSF to other biological properties of B. pertussis (Bergman, Munoz, and Blaskett). Various fractions obtained in the above procedures (except the final agarose electrophoresis purified HSF) have been studied for their ability to stimulate production of lymphocytes (lymphocyte promoting factor, LPF), to induce hypoglycemia and hypoproteinemia, to act as an adjuvant, to induce experimental allergic encephalomyelitis (EAE) in rats, and to protect mice against B. pertussis infection. Although not all the experiments have been completed, the following conclusions can safely be made: 1) Every fraction that contained HSF also had LPF while none lacking HSF activity induced significant lymphocytosis. 2) All fractions that stimulated histamine sensitivity also induced hypoglycemia and hypoproteinemia. 3) Immunity in mice was induced only by those fractions containing HSF, but there was no quantitative correlation between HSF and PA activities. This work is still underway and we expect to have more definite results in the near future. 4) Preliminary tests indicate that only HSF-containing fractions increased the susceptibility of Lewis rats to EAE. 5) Employing the plaque technique of Miller and Warner (Int. Arch. Allergy 40: 59-71, 1971), we found that all fractions with HSF so far tested increased the number of antibody forming cells in the spleens of mice. All fractions that had HSF also increased the production of reaginic type antibodies in mice.

3. Mechanism of action of HSF (Bergman).

a. Histamine sensitivity in mice receiving various agents. A number of compounds known to be T-cell or B-cell stimulators induced a sensitivity to histamine when given IV or IP to mice. This sensitivity is demonstrable 3 hours after administration of the compound but not 3 days later. This phenonemon is clearly different from the effects of HSF, which are long lasting. As little as .016 µg of endotoxin, 4 µg of Poke weed mitogen, and from 50 to 100 µg of phytohemagglutinin or concanavallin A

induced this short-term sensitivity. The activity of these substances, except concanavallin A, was retained after heating at 80°C for 1/2 hour. Crude B. pertussis extracts also have a heat resistant substance (most likely endotoxin) that induces histamine sensitivity 3 hours after administration, but not 3 days later. The increased sensitivity to histamine induced by endotoxin was not due to a pyrogenic effect, since mice treated with endotoxin actually experienced a decrease in body temperature.

b. Vascular permeability changes induced by BPE. Increased permeability of vascular bed has been suspected to be one of the actions of HSF, but it has been difficult to demonstrate. Employing the method of Leibowitz and Kennedy (Immunology 22: 859-869, 1972), we studied changes in permeability of the vascular bed of the mouse thigh muscle. Mice were given 20 µg BPE IV and 2 to 3 days later received $^{131}$I HSA. During the following 24 hours about 50% more labelled antigen leaked from the vascular bed in the BPE-treated than in the saline-treated mice. The possibility of using ultrastructural tracers such as horseradish peroxidase or catalase to study permeability changes is also being explored.

c. Quantitation of catecholamines. Efforts have been continued to develop a highly sensitive method to assay for catecholamines.

4. Effect of HSF on slow virus infection (Munoz, Hadlow, and Bell). Extracts of B. pertussis containing HSF increased the survival of mice to rabies virus given IP. In mink infected with Aleutian disease virus, B. pertussis extracts had no detectable effect on the course of AD in either Sapphire or pastel mink.

Significance to biomedical research and the program of the Institute:

As stated last year, interest in the various biological effects of B. pertussis is increasing as more workers become familiar with the various activities of the organism. Thus, various reports have been published about the lymphocytosis promoting effect of B. pertussis associated with increased susceptibility to EAE and with the increased production of γE type of antibody. We justify our extensive efforts to purify the materials responsible for these effects in order to determine their exact physical and chemical nature. The work that has been done this year indicates that most of the important biological effects of B. pertussis cells may be due to a single substance. This substance has now been obtained free of all detectable contaminating antigens and is active in submicrogram quantities. The exact nature of this material will have to await production of sufficient amounts of material for the required chemical assays.

The substance involved in protecting mice against infection with B. pertussis may not be identical to the HSF because the relationship between HSF activity and protection found in the various fractions is qualitative, but not quantitative. Protection tests are still underway

with the purest HSF preparations and we plan to definitely establish whether HSF does protect or if it acts entirely as a necessary adjuvant for the protective antigen(s).

The mode of action of HSF in its various activities is of great theoretical and possibly practical interest.

B. pertussis extracts have been found active as adjuvants to rabies vaccines and this practical aspect of the problem may prove useful in veterinary and possibly human vaccination.

Proposed course of project:

We shall continue the studies on the various activities of HSF with special emphasis on 1) complete identification of HSF, 2) mode of action of HSF, 3) mode of action of HSF in increasing γE production susceptibility to EAE and production of antibody in general, and 4) the mechanisms by which B. pertussis vaccines protect mice in an effort to identify the protective antigen(s).

Serial No. NIAID-185
1. Rocky Mountain Laboratory
2. Epidemiology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Agents of the psittacosis-lymphogranuloma-trachoma group (Chlamydia)

Previous Serial Number: Same

Principal Investigators: R. N. Philip and R. K. Gerloff

Other Investigators: E. A. Casper and L. A. Thomas

Cooperating Units: Prof. Barrie R. Jones and Dr. John D. Treharne, Institute of Ophthalmology, Univ. of London, England
Dr. John L. Decker, NIAMD, NIH, Bethesda, Maryland
Dr. Carlos Lopez, University of Minnesota, Minneapolis
Dr. Peter Reeve, University of London, England

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 4 2/12 | | | 4 2/12 |
| Professional: | 2 2/12 | | | 2 2/12 |
| Other: | 2 | | | 2 |

Project Description:

Objectives:

The general purpose of this project is to investigate relationships among the chlamydiae, to clarify the epidemiology of chlamydial diseases and determine their total importance to human health, and to apply technological advances to the laboratory study of these organisms.

Specific objectives include: 1) improved methods for isolating chlamydiae from clinical specimens, 2) isolation and characterization of new strains, 3) more luxuriant growth in cell cultures, 4) more sensitive and specific diagnostic reagents and techniques, 5) antibody surveys to

determine prevalence of chlamydial infection in specific human and animal populations, 6) etiologic relationships of chlamydiae to human diseases of venereal origin, and 7) chlamydial interrelationships and characteristics of possible taxonomic value.

Methods employed:

A wide variety of techniques are employed, many of which are standard for chlamydial research. Serologic procedures, including the radioisotope precipitation (RIP) and micro-immunofluorescence (micro-IF) tests,have been emphasized in the past year as ancillary measures to isolation of chlamydiae in elucidating the extent and characteristics of human infection.

Major findings:

1. Serologic surveys. Additional groups of sera were tested in further efforts to define the parameters of RIP and micro-IF tests and the extent of chlamydial infection in various population groups. Included were some 300 sera obtained by Dr. Reeve from gonococcal (GU) and non-gonococcal urethritis (NGU) patients. Chlamydial isolation had been attempted from these persons and over 100 were positive. Also included were 184 sera from 2 groups of Minnesota children (average age 6.8 and 1.8 years) who had central nervous system or other illness but no particular history of chlamydial infection.

In the urethritis group, 94% of patients had RIP antibodies and 85% had micro-IF antibodies at the time chlamydiae were isolated. Comparable figures on the chlamydiae-negative group were 82% and 55%, respectively. Thus, presence of antibody correlated with recovery of chlamydiae, but the high reactivity in both groups was more impressive than the differences, reaffirming our belief that chlamydial infections are frequent in sexually promiscuous populations.

In the older group of Minnesota children, 20% of sera were reactive in the RIP test and 22% by micro-IF, whereas 15% of sera obtained from younger children were reactive in both tests. Thus, at age levels where sexual activity was low or nonexistent, serologic reactivity was likewise low. Nevertheless, for reasons unknown, several sera from young children did have substantial titers in both tests.

To gain still further information on the relationship of age to serum reactivity, all specimens submitted to RML by practitioners in this area for routine serodiagnosis in 1971 were tested for chlamydial antibodies by micro-IF. Of 91 sera from patients suspected to have spotted fever, Colorado tick fever, or leptospirosis, 38% had antibodies. Although the numbers are small, the patterns of reactivity by age corresponded to expected patterns of sexual activity by age. Thus, 20% of children, 47% of the 15-29 year group, 42% of the 30-39 group, and 35% of those over 49 were reactors.

Sera from a few laboratory-confirmed cases of rickettsial disease, psittacosis and Colorado tick fever were also tested for chlamydial antibodies by micro-IF. There did not appear to be appreciable cross-reactivity in nonchlamydial illnesses.

2. Comparison of RIP and micro-IF tests. Over 800 sera have now been tested by these procedures and correlation between the 2 tests has been consistently good in all study groups. The RIP test is more sensitive than the micro-IF test. Thirty-three percent of sera negative by micro-IF were weakly positive by RIP, whereas only 10% of those negative by RIP were positive by micro-IF. On the average, RIP titers were 6-fold higher than micro-IF titers.

The RIP test measures chlamydial-group antibody when meningo-pneumonitis (Mpn) is used as antigen. The micro-IF test detects both group and type-specific antibody. In sera where no type-specific antibody can be detected, the RIP test is considerably more sensitive than the micro-IF test. In sera where type-specific antibody is also present, the RIP test is only slightly more sensitive than the micro-IF test. Using antiglobulin prepared against human globulin undifferentiated as to immunoglobulin class, the 2 tests probably measure antibodies predominantly of the IgG class. Anti-IgG is now being used routinely in RIP tests.

A handicap with the micro-IF test is that endpoints are subjectively determined and reliability of the test is dependent on experienced readers. RIP endpoints are objectively measured. Nevertheless, both tests are highly reproducible. In tests of serum pairs, antibody titers and patterns of reactivity were remarkably uniform.

3. IgM antibody. IgM antibody increasingly appears to be a useful indicator of current infection and reflects nature of the infecting serotype. In Dr. Reeve's urethritis patients, 27% of the chlamydiae-positive group had IgM antibodies detected by micro-IF using $F_c$-specific anti-IgM conjugate. In contrast, only 11% of the chlamydiae-negative group were reactors. Nearly all IgM reactive sera showed patterns of type-specificity. We did not have Dr. Reeve's isolates to match against the corresponding sera, but previous work has indicated that the serotype of the infecting strain does correspond with the patterns of IgM antibody response.

4. Use of strains other than Mpn in RIP tests. $P^{32}$-labeled Mpn particles propagated in L cells have been routinely used in RIP tests. Because human strains might be advantageous as antigens, continued attempts were made to prepare antigen from LGV (strain 404) and inclusion conjunctivitis (strain LB-1) chlamydiae grown in Hela 229 cells. Results have continued to be poor. Growth of LB-1 in L cells will be tried soon in case the Hela cells were contributing a deleterious factor.

5. Quantitation of globulin-antiglobulin precipitate in RIP tests. In order to determine whether antigen particles may be nonspecifically adsorbed or trapped in large precipitates, the precipitates from tests representing sera of varying antibody titers obtained from children and adults were quantitated. In adult sera, negative specimens in dilution 1:32 produced the same amount of precipitate as strongly positive sera. In children's sera, a direct relationship existed between age of the donor and amount of precipitate per test. However, only with sera from children less than 6 months old was the amount below the range considered normal for adult sera. In titrations of positive and negative sera, large amounts of precipitate could be accompanied by minimal precipitation of radioactivity. Also, with increasing dilution of serum, the percent radioactivity precipitated and the amount of precipitate did not decrease at the same rate. These data indicate that nonspecific adsorption is not an important factor in precipitating radioactivity.

6. Blocking of the RIP reaction by unlabeled chlamydiae. In experiments to get a better understanding of the nature of the antigen-antibody reactions in the RIP test, suspensions of Mpn, LGV (404), LB-1 and mouse pneumonitis (Nigg II) were used to block the Mpn-$P^{32}$ antigen-antibody reactions. Results to date indicate that significant blocking requires very high concentrations of antigen and that only a small portion of the combining capacity of antibody is actually utilized in the routine RIP test. Although strains appear to be different in blocking efficiency, this may have been due to variability in concentrations of particles rather than antigenic differences in strains.

Significance to biomedical research and the program of the Institute:

In recent years less public health attention has been given to diseases caused by chlamydiae indigenous to animals and birds and more directed toward diseases caused by agents which occur only in man. These include with certainty the eye diseases, trachoma and inclusion conjunctivitis, and the venereal disease, lymphogranuloma venereum. The evidence is now quite convincing that a large reservoir of genital infection exists and that many different serotypes of trachoma-inclusion conjunctivitis chlamydiae may be venereally transmitted. Their relationships to various diseases of the genito-urinary tract such as nonspecific urethritis and Reiter's syndrome continue to be important but still largely unanswered questions.

The studies described above were designed to contribute, in one way or another, to a better understanding of these relationships and the relationship between ocular and genital chlamydiae in general.

Proposed course of project:

Although we will continue to investigate basic laboratory questions related to serologic and primary isolation techniques, we now feel that we have progressed to a point where increasing emphasis can be directed toward application of the diagnostic procedures in field investigations. Collaboration, particularly with Dr. Reeve, has been fruitful and it will continue. However, we also believe that similar studies should be initiated in this country. Several possibilities are being considered. One involves a multi-team approach to determining the etiologic relationship of chlamydiae to NGU in San Francisco, Seattle, Boston, and the District of Columbia. We would participate as one of 4 groups studying this problem and would organize and carry out the investigation in Washington, D.C. Another possibility is a long-range, broader, independent study in an urban area, such as Washington, involving collaboration of clinicians in gynecology and urology, as well as V.D. clinics, aimed at determining the relationships of chlamydial infection to such problems as NGU, cervicitis, salpingitis, prostatitis, spontaneous abortions, stillbirths, and prematurity. Serologic procedures would be used to detect potential associations and isolation techniques plus strain typing and serologic responses for definitive characterization of etiologic relationships.

Publications:

Page, L. A., Frey, M. L., Ward, J. K., Newman, F. S., Gerloff, R. K., and Stalheim, O. H.: Isolation of a new serotype of mycoplasma from a bovine placenta. J. Am. Vet. Med. Assoc. 161: 919-925, 1972.

In press:

Hill, D. A., Philip, R. N., Greaves, A. B., and Purcell, R. H.: A study of mycoplasmas in urethritis patients attending a venereal diseases clinic. Brit. J. Vener. Dis.

Gordon, F. B., Quan, A. L., Steinman, T. I., and Philip, R. N.: Chlamydial isolates from Reiter's syndrome. Brit. J. Vener. Dis.

Serial No. NIAID-186
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The biology of microbial agents in arthropods

Previous Serial Number: Same

Principal Investigators: W. Burgdorfer and L. P. Brinton

Other Investigators: R. L. Anacker, R. K. Gerloff, and L. A. Thomas

Cooperating Units: NAMRU-3, Cairo, Egypt
Major D. L. Huxsoll, Division of Veterinary Medicine, Walter Reed Army Institute of Research
Dr. B. N. Chaniotis and Dr. R. B. Tesh, MARU
Dr. R. H. Wharton, CSIRO, Indooroopilly, Queensland, Australia

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 2 9/12 | | | 2 9/12 |
| Professional: | 1 4/12 | | | 1 4/12 |
| Other: | 1 5/12 | | | 1 5/12 |

Project Description:

Objectives:

To study dynamics of viral, rickettsial and bacterial infections in arthropod vectors and the interactions between microorganisms and arthropod host, as reflected by changes in morphology, histology and fine structure of arthropod tissues. Specifically investigated are 1) development of pathogens within growth-supporting cells, 2) cellular response(s) to infection, 3) effects on physiologic characteristics resulting from pathogen-arthropod association, and 4) factors supporting or inhibiting development and survival of infectious agents.

Research during the past year included 1) vesicular stomatitis virus (VSV) growth and cellular interaction in phlebotomine sandflies, 2) Colorado tick fever (CTF) virus morphogenesis and localization in Dermacentor andersoni tissues, 3) study of rickettsialike nonpathogenic microorganisms in field-collected Amblyomma americanum and Ixodes scapularis, 4) isolation and characterization of bacilluslike organisms from Dermacentor ticks, 5) isolation and characterization of Wolbachialike tick symbiotes, 6) vector-pathogen relationship of Ixodes holocyclus and Rickettsia australis, 7) development of Ehrlichia canis in Rhipicephalus sanguineus and D. variabilis, 8) detection and development of Trypanosoma theileri in Ethiopian cattle ticks, and 9) fine structure studies related to spermatogenesis in D. andersoni.

Methods employed:

In addition to routine techniques of medical entomology, bacteriology, and virology, fluorescent antibody procedures are applied to studies of viral, bacterial, and rickettsial infections in arthropods. Electron microscopy is used to characterize subcellular structures of normal and infected arthropod tissues and to determine morphology of microorganisms during intracellular development.

Major findings:

1. In cooperation with Drs. Chaniotis and Tesh, MARU, tissues of approximately 400 phlebotomine sandflies experimentally infected with VSV have been processed for electron microscopy and await sectioning and examination.

2. No success has been realized in demonstrating CTF virus in ultrathin sections of tissues from infected D. andersoni nymphs. To assure heavily infected ticks, a new experiment was initiated in which ticks were fed on viremic golden-mantled ground squirrels (Citellus lateralis tescorum) and/or chipmunks (Eutamias spp.) during their larval, nymphal and adult stages. Several engorged females which proved infected with CTF virus by suckling mouse test have been processed for electron microscopy and await examination.

3. A rickettsialike organism related by immunofluorescence to the spotted fever group but nonpathogenic for laboratory animals was detected in 64 of 545 adult A. americanum from "Land Between the Lakes", and in 194 of 482 ticks of the same species collected in Arkansas. Its fine structure indistinguishable from pathogenic rickettsiae, this microorganism was found in rather moderate quantities in all tick organs, and was passed trans-ovarially by infected females to 100% of filial ticks. Isolation attempts were uniformly negative in embryonated hens' eggs but were successful in primary chick embryo tissue cultures where plaques similar to those of spotted fever rickettsiae were produced. Injection of concentrated tissue

culture material into meadow voles and guinea pigs did not produce infections detectable either microscopically or serologically.

A rickettsialike microorganism related by immunofluorescence to both the spotted fever and typhus groups of rickettsiae was noted in 7 of 102 Ixodes scapularis from Arkansas. Massively distributed throughout all tick tissues, this organism too proved noninfectious for laboratory animals.

4. Several isolations of bacilluslike nonpathogenic microorganisms (not Wolbachia) from D. andersoni (western Montana) and D. variabilis (Kentucky and Tennessee) have been accomplished by inoculation of infected tick-hemolymph into primary chick embryo tissue cultures. Plaques formed by these organisms differed in many respects from those produced by pathogenic rickettsiae.

5. Attempts to grow Wolbachialike symbiotes of ticks on primary chick embryo tissue cultures have been successful. Isolations have so far been accomplished from D. andersoni, D. variabilis, A. americanum, and A. variegatum. These symbiotes, like rickettsiae, form plaques.

6. Initial attempts were made to infect I. holocyclus, supplied by Dr. R. H. Wharton, CSIRO, Queensland, Australia, with R. australis, the agent of Queensland tick typhus. However, due to enormous difficulties in maintaining this tick in the laboratory, we have not been able to establish an infected tick line. Studies are in progress to determine biologic and physiologic conditions necessary for successfully rearing this tick.

7. Additional attempts were made by Dr. Huxsoll, WRAIR, to infect R. sanguineus and D. variabilis with Ehrlichia canis by feeding ticks on parasitemic dogs. Again, we failed to detect the agent by conventional and fluorescence microscopy in any of the ticks supplied. Therefore, ticks were infected by intracelomic injection of E. canis grown on dog monocyte cell cultures. No evidence has been obtained so far that E. canis developed in tick tissues.

8. Trypanosoma theileri was detected in 19 of 258 adult Rhipicephalus pulchellus and in 1 of 69 Boophilus decoloratus taken off cattle in Ethiopia. All developmental stages were found in Giemsa-stained smears prepared from hemolymph and various organs of these ticks. The ticks' body fluid appeared to provide optimal conditions for massive multiplication of epimastigotes whereas all tissues, but particularly ovary, were heavily infected with organisms ranging from amastigote to trypomastigote stages. The presence of trypomastigotes in salivary gland tissues of both R. pulchellus and B. decoloratus suggests that these ticks may transmit this organism by bite. Despite massive invasion of ovarian tissues, there was no evidence of transovarial passage of T. theileri to the progeny of infected R. pulchellus.

9. In line with our efforts to obtain a thorough knowledge of the various biologic and physiologic processes in development of tick vectors, a fine structure study on spermatogenesis in D. andersoni was initiated. Accordingly, spermatogonia were seen partitioned into packets or cysts by a simple squamous epithelium; they exhibited very little organellar differentiation and apparently are in a state of latent activity prior to attachment of the male to a host. Twenty-four hours after attachment, spermatogonia in cysts at the posterior region of the paired gonads enlarge synchronously to form primary spermatocytes. Development proceeds from a posterior to anterior direction in both testes. All germ cells in a given cyst are interconnected by intercellular bridges and development is synchronous through the completion of both meiotic divisions. Primary spermatocytes possess prominent nucleoli, form abundant Golgi bodies or dictyosomes that are dispersed throughout the cytoplasm and form shallow to rapidly deepening U-shaped membranous cisternae.

Significance to biomedical research and the program of the Institute:

The potential medical and veterinary importance of phlebotomine sandflies as both vectors and reservoirs in the transmission and maintenance of VSV have been established. Observations from our study will provide not only information regarding developmental fine structures and cellular interactions of VSV in sandflies, but also should help clarify the mechanisms of ovarial infection—a phenomenon unusual for virus-infected Diptera.

Development and intracellular growth of CTF virus may follow a pattern different from that established for other arboviruses. This is suggested by detection (Emmons, 1972) of distinct fibrillar bodies in association with this agent in mouse erythrocytes and BHK-21 cells in vitro.

Presence in A. americanum and in I. scapularis of rickettsialike microorganisms related by immunofluorescence to one or several rickettsial groups suggests existence in nature of unknown rickettsiae that may be pathogenic to man. Since both species of ticks readily feed on man, characterization of these microorganisms is essential.

Successful isolation and culturing of the bacilluslike microorganisms from Dermacentor ticks will provide the material necessary to study the biochemical composition of these organisms and their relationship to rickettsiae in general and to the spotted fever group in particular.

Primary chick embryo tissue cultures provide an ideal tool for the isolation of tick symbiotes and enable us to investigate the poorly understood biologic and physiologic functions of these organisms. Also, it is now possible to determine the nature and extent of antigenic relationships among tick symbiotes.

Queensland tick typhus in Australia is probably more common than medical reports indicate. On circumstantial evidence, I. holocyclus has been incriminated as the vector of the causative agent, R. australis. Since all stages of this tick attack man readily, information concerning their ability to maintain and transmit R. australis should be available.

Failure to infect ticks with E. canis, either by feeding on sick dogs or by injection of the agent into ticks' hemocele, is puzzling in view of the consensus that ehrlichiosis is a tickborne disease of dogs. However, it may be possible that development of E. canis in ticks is obscured by the presence of Wolbachialike symbiotes which show a remarkable morphologic resemblance to this agent. Canine ehrlichiosis has been reported in this country, and it appears quite possible that the American dog tick, D. variabilis, in addition to R. sanguineus may serve as a vector and reservoir.

Trypanosoma theileri, considered to be transmitted to cattle by tabanid flies, has been suggested as the cause of disease or debility in cattle, either alone or in association with protozoan, bacterial, and viral agents. Detection of this flagellate in cattle ticks from Ethiopia, its luxurious growth in tick hemolymph and tissues, but particularly the presence of trypomastigotes in salivary glands, suggest that ticks are indeed involved in the transmission and maintenance of this organism among cattle.

Our data related to spermatogenesis, like those of spermiogenesis and sperm behavior in the female reproductive tract (see Annual Report 1971/72), provide the basis for evaluating the hypothesis that gametes of rickettsia-infected ticks may transfer infection to normal females and infect eggs as well as offspring.

Proposed course of project:

Because technical assistance has not been available in the electron microscopy unit during the past year, considerable time had to be devoted to the preparation of incoming research material. Consequently, progress has been rather slow. The vesicular stomatitis virus and Colorado tick fever virus projects will receive priority during the coming year. It is also hoped that some efforts can be directed toward the long programmed clarification of "sexual transmission" of spotted fever rickettsiae in D. andersoni.

Attempts will be made to obtain purified preparations of the rickettsialike microorganisms from A. americanum and I. scapularis, as well as of the bacilluslike organisms from Dermacentor ticks to determine growth characteristics, DNA composition, antibiotic susceptibilities, and, where pertinent, the biochemical and immunologic relationships to pathogenic rickettsiae.

In addition to electron microscopy to establish morphology and cellular relationship of various tick symbiotes, studies into their metabolic activities are planned. Cellular incompatibility in ticks resulting from experimental Wolbachia infection and the use of this phenomenon in tick control will be investigated.

Once rearing conditions for I. holocyclus have been worked out, studies pertaining to the development and behavior of R. australis in this tick will be resumed.

Further attempts will be made to infect lines of R. sanguineus and D. variabilis with E. canis; dogs will be used to determine tick infectivity.

It is hoped to provide a graduate student in the Zoology Department of the University of Montana with Trypanosoma theileri and R. pulchellus to conduct a dynamic study with detailed histologic examination to establish the tick tissues involved in the cyclical development of this agent, and to determine capability of ticks to transmit this trypanosome by bite.

Honors and Awards:

Dr. Willy Burgdorfer

Continued to serve as Consultant to University of Maryland Medical School (Dr. Charles L. Wisseman, Jr.) on contract NR-136-889 related to tickborne pathogens and extrahuman typhus infections.

Continued to serve as Faculty Affiliate for the Department of Zoology, University of Montana, Missoula.

Dr. L. P. Brinton

Served as a discussant and presented paper in the Symposium on Reproduction of Arthropods of Medical and Veterinary Importance at the XIV International Congress of Entomology, Canberra, Australia, August 22-30, 1972.

Publications:

In press:

Burgdorfer, W., Brinton, L. P., and Hughes, L. E.: Isolation and characterization of symbiotes from the Rocky Mountain wood tick, Dermacentor andersoni. J. Invertebrate Pathol.

Burgdorfer, W., Schmidt, M. L., and Hoogstraal, H.: Detection of Trypanosoma theileri in Ethiopian cattle ticks. Acta Tropica

Serial No. NIAID-187
1. Rocky Mountain Laboratory
2. Zoonoses Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Host-ectoparasite relationships

Previous Serial Number: Same

Principal Investigator: J. F. Bell

Other Investigators: C. M. Clifford and J. E. Keirans

Cooperating Units: Dr. W. A. Nelson, Canada Department of Agriculture Research Station, Lethbridge, Alberta

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 9/12 | | | 9/12 |
| Professional: | 1/12 | | | 1/12 |
| Other: | 8/12 | | | 8/12 |

Project Description:

Objectives:

Studies are directed toward classification of certain phases of host/arthropod relationships. Emphasis is centered on identifying the mechanisms by which animals become resistant to feeding of arthropods and factors governing responses of parasitized animals to infection with various microorganisms.

Methods employed:

The host-parasite system developed at the RML whereby measurable and predictable populations of lice can be developed on various strains of mice by restriction of self-grooming is extensively used. At times other laboratory animals are utilized and a variety of microbiologic, immunologic and entomologic techniques are employed.

Major findings:

Absence of Mr. Hestekin for much of the year because of training activities and transfer has limited activity on this project. Two studies were pursued, neither one to conclusion.

1. Protection of animals vs. an arthropod vectored pathogenic agent (Francisella tularensis) by acquired hypersensitivity to the vector. Rabbits were sensitized by feeding normal Dermacentor andersoni nymphs on their ears. Ticks were infected, as larvae, by feeding on rabbits infected with virulent F. tularensis type A. Large numbers of heavily infected flat nymphs were then placed on sensitized and normal rabbits. In this test the sensitized rabbits became infected, as did the controls, but death was delayed in the sensitized group. We believe that overwhelmingly severe challenge accounts for the mortality in the sensitized group.

2. Induction of tolerance to lice in white mice. Newborn mice were subjected to heavy louse feeding repeated at frequent intervals. In another group, newborn mice received parenteral inoculations of "solubilized" louse antigen and subsequently received additional injections of that antigen. There was no significant difference in mortality or average louse burdens when the test and control animals were infested with lice. New studies designed to produce continuous exposure to lice from the moment of birth are under way.

Significance to biomedical research and the program of the Institute:

Theoretically, an organism that can be perpetuated by transstadial passage in the vector, and can also be acquired by the vector from hosts, should eventually infect all members of the vector population. Obviously there must be factors that limit perpetuation and transfer because not all vectors or hosts are infected. One observed (by us) limitation is the nonspecific resistance to a vectored agent by virtue of hypersensitivity to the vector. That resistance is not of very high order, but nevertheless may be significant.

Hypersensitivity and immunity of classical kinds have been demonstrated in some host/ectoparasite relationships but the strong resistance to lice developed by mice does not seem to fit any of the established concepts. We are attempting to produce tolerance to lice as much for its probable contribution to understanding of resistance as for its applicability to human or veterinary health.

Proposed course of project:

These studies will be pursued to definite and conclusive ends.

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1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

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Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Vector-pathogen relationships

Previous Serial Number: Same

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Dr. M. N. Kaiser, NAMRU-3, Cairo, Egypt

| Man Years: | NIAID Lab Staff | Other NIH Personnel Assigned to Project | Guest Workers | Total |
|---|---|---|---|---|
| Total: | 2 1/12 | | | 2 1/12 |
| Professional: | 5/12 | | | 5/12 |
| Other: | 1 8/12 | | | 1 8/12 |

Contract Support:

| Contract Number | Project Officer (NIAID) | Contractor | Contractor's Project Director | Annual Funding Level |
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| PL-480 03-016-1 | C. E. Yunker | NAMRU-3 Cairo, Egypt | M. N. Kaiser | $67,594 |

Project Description:

Objectives:

1) To investigate the host-range, cellular relationships and growth dynamics in arthropods or their cells of pathogenic agents transmissible by vectors among man or animals, 2) to develop systems of vector or vector-related tissue and cell culture and to apply these systems to the study of arthropod-borne disease and vector-pathogen relationships, 3) to define the microenvironment to which pathogenic microorganisms are subjected when ingested by tick vectors, 4) to provide basic biochemical information necessary for the progress of developmental tick tissue culture studies.

Methods employed:

Disease agents of primary interest are arthropod-borne viruses and, to a lesser extent, rickettsias, which are transmitted by Acarina and Diptera. Thus, conventional basic techniques in laboratory arbovirology and experimental acarology and entomology are employed. A major effort involves development and use of arthropod tissue and cell cultures. Standard and specially devised biochemical and physiological techniques are used to determine the nature of tick body fluids. Resultant findings are employed in the preparation of tick tissue culture medium in efforts to improve the growth supporting characteristics of the medium.

Major findings:

1. An extensive study of the ability of various viruses to produce plaques in mosquito cell monolayers was completed. Of 124 viruses tested, 30 (24.5%) were found to possess this property. All but two of those positive were classical arboviruses of the serological groups A, B, C, and Bunyamwera. Plaquing ability reflected group relationships and vector class. Only one of 9 A group arboviruses plaqued. Of 33 B group agents, 17 that are mosquito-borne plaqued; the remaining 16, with two exceptions, are either tick-borne or not arthropod-borne. Members of the serologically related Bunyamwera and California groups (all mosquito-borne) were distinguishable by their ability to cause plaques; the former were positive, the latter negative. Fifty-nine miscellaneous viruses failed to plaque. Of these, only 6 are mosquito-borne and the remaining 53 are either tick-borne or not vector-borne. A manuscript describing these results is in preparation.

A mosquito cell line recently established from Aedes w-albus was also investigated for plaque forming ability. Of 20 arboviruses tested, plaques were induced by 5: Dengue-2, Dengue-3, Japanese B encephalitis (B group), Chittoor (Bunyamwera group) and Tribec (Kemerovo group). All of these viruses, which give excellent plaques in the A. albopictus

cell-line, caused poorly defined plaques; they propagated at lower titer, and results were inconsistent. Thus A. w-albus system is less satisfactory than that of A. albopictus.

Successes in plaquing arboviruses in mosquito cells prompted us to attempt rickettsial plaquing in them. With little modification of procedure, two Rickettsia spp., R. rickettsi (R strain) and R. conori (Simko strain) induced the formation of well-defined plaques in A. albopictus cells. Initial attempts to plaque other rickettsias (R. prowazeki, R. mooseri, Coxiella burneti and Pope's rickettsia) were unsuccessful. In support of this study we developed a reproducible plaque assay system for rickettsias in Vero cells. Plaques produced in these cells by R. rickettsi, R. montana, R. conori, R. canada, R. prowazeki and Pope's rickettsia were well-defined and rickettsial growth was high in titer.

2. We continued to investigate aspects of arbovirus growth in arthropod cells grown in vitro. Murutucu, a group C arbovirus which plaques well in A. albopictus monolayers but which fails to do so in A. w-albus cells, was inoculated in parallel into both of these cell-lines and fluid cultures were observed for virus growth by secondary assay in Vero cells. Surprisingly, titers were 1 1/2 logs higher and peak virus production occurred two days sooner in the A. w-albus system. Other viruses tested in various arthropod cell systems were Bakau and Chenuda, Nyamanini and Quaranfil viruses. The first, a mosquito-borne virus, failed to propagate in Antheraea eucalypti (moth) cells, Argas persicus and Dermacentor andersoni hemocytes or primary cultures of D. andersoni viscera, but excellent long term maintenance, indicative of low level proliferation, was seen in A. albopictus cells. The other three viruses, all tick-borne, deteriorated rapidly in the mosquito and moth cells, but, in at least one of the tick tissue culture systems, titers were maintained at relatively high levels, in spite of repeated medium changes, for up to five weeks.

Last year we demonstrated complete and prolonged (but temporary) inhibition of mosquito cell devision by mitomycin. This year we investigated the role of cell multiplication in production of virus, the effect of mitotic inhibition on plaque formation by viruses, and the feasibility of using mitomycin-treated mosquito cells as feeder layers for tick cells (see par. 3). Studies with Colorado tick fever, yellow fever, and Kemerovo viruses indicated that, in general, virus production in fluid overlay cultures is proportionate to cell number, although in both treated and untreated systems virus levels may increase above the amounts inoculated. Mitomycin-treated A. albopictus cells were also infected with C. burneti in the hope that a measurable cytopathogenic effect (CPE) would result from the growth of rickettsias surpassing the available cell units of substrate. Instead, only slight CPE was seen and multiplication of the rickettsia was markedly less than in untreated controls. In addition, infection of the treated cells, as opposed to untreated ones, was

characterized by a chain-like morphology of the rickettsia and an absence of syncytia of host cells. All of these observations, in one way or another, reflect the influence of the dividing nucleus on proliferation and cell destruction by this characteristically cytoplasmic rickettsia.

Because arbovirus infections of mosquito cells in fluid culture are characteristically inapparent and infectious center studies reveal only a small proportion of cells to be infected, it is desirable to determine what causes the plaques to form in the overlay system. We compared growth of 7 arboviruses in A. albopictus monolayers in fluid medium and in agar overlay medium. Despite the restriction of virus dissemination in agar-overlay cultures, as much virus was recovered from these as from fluid cultures. This may indicate that cells not otherwise destined to become infected, when overlayed, are involved in virus production. Additional factors will be studied.

3. In continued efforts to establish an in vitro line of cells from ticks the most promising results to date were obtained with the serial cultivation, for up to four passages, of large numbers of hemocytes. This progress resulted from our previously reported development of a valid method for cultivation of hemocytes plus media modifications. Insight for the latter was gained in part from results of our PL-480 contract with NAMRU-3. Tick hemocytes were also cultivated on mitotically inhibited feeder layers of mosquito cells in attempts to produce a line of serially replicating tick cells. Up to seven subcultivations were made, but since the effectiveness of mitomycin is eliminated at this level, identity of the cells - whether tick, mosquito or hybrid - becomes moot.

In other developmental tissue culture aspects, two morphological cell types of our previously reported substrain of serum-free A. albopictus cells were recognized and one of these was successfully purified by cloning. Its growth curves, in comparison to the parent strain and other populations, were investigated.

4. Arboviruses are not known to induce haemadsorption activity or syncytial formation in vertebrate cells. Because they commonly induce syncytia in mosquito cells in culture and because a haemagglutinin (HA) had been reportedly found in "normal" A. albopictus cells, we investigated the possibility that arbovirus infection might elicit haemadsorption activity or an HA in mosquito cells. If so, additional means with which to identify arboviruses would become available. In three experiments, uninfected A. albopictus cells and cells infected with 1 of 8 different arboviruses were examined for these activities by conventional techniques employing various pH's, temperatures and species of red cells. Results were uniformly negative.

5. This year we isolated an agent from a line of Anopheles stephensi cells sent to us by Dr. H. Hirumi (Boyce Thompson Institute).

This agent induces formation of syncytia in these cells and in those of A. albopictus as well. The agent was identified as a filterable virus serologically closely related to Chikungunya and O'nyong-nyong viruses. In A. albopictus and Vero cells the isolant could be plaqued only at low temperatures (27-32°C) and its multiplication, as expressed by CPE, was greatest at lowest temperatures of incubation. It is most likely a cross-contaminant of Anopheles stephensi cells since the infected cell-strain was obtained by Dr. Hirumi from a laboratory in which a Chikungunya-like virus was known to have been latently carried in mosquito cells. It is of interest to us because of the implied possibility that a temperature-sensitive variant of an arbovirus may have evolved or been selected as a consequence of its existence in a vector-cell system. The isolant is being characterized physically and biologically at RML and by electron microscopy at Dr. Hirumi's laboratory.

6. A search for interferon or viral interference in insect cells was continued in collaboration with Dr. Samuel Baron (LBV). Singh's A. aegypti cells were exposed to the interferon inducer polyriboinosinic-cytidylic acid (poly I·C) and challenged with the interferon-sensitive Sindbis and Semliki Forest viruses. Culture fluid samples taken at intervals and assayed in Vero cells revealed no reduction of virus yields of poly I·C treated cells over untreated controls. Thus evidence is accumulating that an interferon-mediated defense system, as known for vertebrates, is lacking in these invertebrates.

7. As in past years, we have continued to supply, upon request, cell cultures, consultation and information to institutions and investigators outside the Institute. Of particular interest was the isolation and characterization of two Herpesvirus strains from brain material which the Montana State Board of Health obtained from three fatal cases of human encephalitis.

At RML we have provided tissue culture support to projects other than this (see NIAID-180, 181, 182). For example, serological reagents were prepared from tissue culture materials, the course of human infection with CTF virus was followed in tissue culture as an adjunct to mouse studies, and procedures were developed with which to increase the rate of isolation of tick-borne viruses from clinical materials.

8. Gratifying progress was made in tick biochemistry and physiology studies under PL-480 Agreement No. 03-016-1 as evidenced by the publication of 22 research articles. Some of these were reviewed as articles in press or graduate theses in earlier reports. Additional significant findings now published include:

a. Radiotracer studies of various phases of ingestion, digestion and excretion in fed nymphs of Argas arboreus have pinpointed cellular mechanisms and sites for these processes.

b. Studies of seasonal activity, feeding behavior and survival rates of A. arboreus in a natural situation have demonstrated the deleterious effects on populations of A. arboreus of the annual departure of migratory bird-hosts of this species. Although laboratory studies had previously shown the adult ticks' ability to survive for over a year without feeding, larvae and early nymphs were seen to disappear and oviposition by impregnated females ceased with the departure of the host from natural habitats. Obviously the ecology of arboviruses and other pathogens transmitted by these ticks would also be affected.

c. Events of the meiotic cycle of ixodid ticks were documented. In these forms sexual differentiation is recognizable as early as the unfed larval stage.

d. Concentrations of various lipid classes were found to differ more between different physiological states of a single tick species than between species of a given genus. Absence of a pathway for sterol biosynthesis is suggested for both argasid and ixodid ticks. Lipids are significant energy donors in these ticks and sterols in particular are important as growth regulating hormones and as components of the water-regulatory cuticular waxes. These findings imply a universality of lipid metabolism among tick families and, for sterols, between ticks and insects, thus helping to simplify the formulation of an optimum tick tissue culture medium.

e. Morphological markers identifying the physiological stage and age of ticks were used in studies of lipid composition and as a basis for the study of respiration of Hyalomma ticks and hemolymph volumes (absolute and percent body weight) of Argas ticks. Recognition of these markers is an essential preliminary to comparative studies of physiology, biochemistry and water balance among families, species and even individuals of ticks.

f. Hemolymph proteins of both argasid and ixodid ticks were found to characteristically be lipid-conjugated and, to a lesser extent, carbohydrate-conjugated. Differences between the two genera of ticks, but not between species of a single genus, were seen which probably reflects the markedly different types of feeding behavior and development between soft and hard ticks.

g. Previously guanine was identified as the main nitrogenous end product of argasid metabolism, which implies the employment by these ticks of relatively inefficient high energy (energy rich phosphate bond) systems. In ixodids, the identification of a second, previously undescribed, purine compound came as a surprise. Further study of this substance may shed some light on pathogen-vector relationships, since it is possible that the compound is a symbiont-byproduct that is excreted by ixodid and retained by argasid ticks.

h. A nearly complete quantitative analysis of individual amino acids in the hemolymph of an argasid species provides us with significant insight into the composition of an ideal tick tissue culture medium. Although a high and relatively constant amino-acidemia was seen in hemolymph, levels of amino acids were much lower than in insects and are adjudged insufficiently high to play an important role in the regulation of hemolymph osmotic pressure.

i. A high Na/K ratio was found to exist in both argasid and ixodid hemolymph. This ratio is typical of bloodsucking insects, as opposed to phytophagic ones in which the opposite is true. A remarkably high K concentration in the gut fluid of argasid ticks may reflect the presence of a Na/K pump between gut lumen and hemolymph. Higher Na and Cl concentrations in hemolymph and coxal or molting fluids of argasid and ixodid ticks, respectively, than in gut content demonstrate the regulatory function of these ions. Hemolymph osmotic pressures, together with a relatively low amino-acidemia, indicate that ionic composition is the major determinant of total hemolymph osmolarity.

j. Biochemical methodology has also profited by "spin-offs" resulting from these studies. A simple method for the quantitative determination of proline in biological fluids was developed; in this isotin is used as a color reagent. Existing methods are complicated by the interference of other amino acids and peptides. In addition, a new sensitive, convenient method, employing phosphoric acid, was described for the detection and separation of indole derivatives in thin layer chromatography. The 18 standard amino acids, except tryptophan, gave no color with the reagent. Tryptophan, indole and indole-3-acetic acid produced characteristic colors upon treatment.

Significance to bio-medical research and the program of the Institute:

Demonstration of the wide applicability of the arthropod cell-virus plaquing technique provides an additional resource for the isolation, identification, and quantitative assay of various arboviruses. The classification of arboviruses, which is presently tentative and largely artificial, could conceivably benefit by utilization of plaquing reactions. Plaque production, which is an expression of pathogenicity, was shown here to correlate well with the class of vector an arbovirus is adapted to utilize. This would indicate that these pathogens of vertebrates are also less than benign in their invertebrate hosts. Possibly arboviruses, like other parasites, exist in a delicate state of balance with their environment in these hosts, depending upon a critical control for their propagation and transmission. Elucidation of the mechanism involved could provide a means for turning the virus against its vector. One such mechanism may be cellular division. Experiments with mitotically inhibited mosquito cells provide evidence that mitosis is necessary for high levels of pathogen production and in the case of C. burneti, for large scale production of CPE.

The development of techniques for the cultivation and quantitation of rickettsias increases the number of tools available for work in rickettsial diseases.

Analytical studies of the biochemical composition of tick body fluids are essential to meaningful efforts in developmental tick tissue culture, and in addition, provide knowledge of factors necessary for survival, propagation and transmission of tick-borne pathogens.

Proposed course of project:

Characterization of arboviral and rickettsial growth in arthropod cells, with emphasis on expression of pathogenicity, will continue. Refinement of mammalian tissue culture systems as assay tools for this project and for the production of serological reagents for other projects will be continued. Our search for interferon or interference-like phenomena in arthropod cells will be terminated this year.

Developmental tick tissue culture efforts are presently hampered by the loss of our only insectary attendant. Also, our PL-480 contractors overseas have relied heavily on our ability to produce certain of the ticks used in their biochemical studies. Although the latter program will be funded until mid-1973, its results will be less applicable to our developmental tissue culture project than anticipated. Until such time as authorization to replace our insectary attendant is made, both of these projects will be affected.

Honors and Awards:

Dr. C. E. Yunker

Served as guest lecturer at the W. Alton Jones Cell Science Center, Lake Placid, N.Y.

Continued as Affiliate Professor of Zoology at the University of Montana.

Reviewed research manuscripts for the Journal of Parasitology, Journal of Medical Entomology, Entomological Society of America and reviewed grant proposals for the Division of Research Grants, Tropical Medicine and Hygiene Study Section.

Continued as member of Editorial Board, Journal of Medical Entomology.

Publications:

Parker, R., Ribi, E., and Yunker, C. E.: Application of centrifugal chromatography to the separation of sugars in arthropod hemolymph and tissue culture medium. In Rodriguez, J. G. (Ed.): Insect and Mite Nutrition. Amsterdam, North-Holland Publishing Co., 1973, pp. 397-406.

In press:

Yunker, C. E. and Cory, J.: Growth of Rickettsia tsutsugamushi in insect cells. In Proc. 3rd Int. Coll. on Invert. Tissue Culture, Smolenice, 1971.

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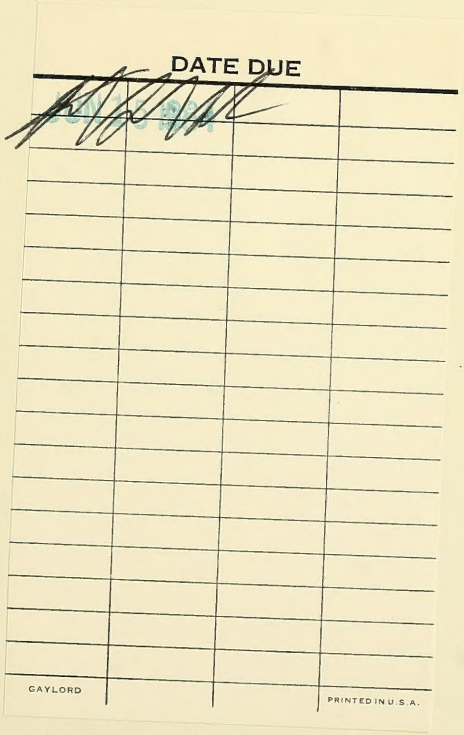


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